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**Lipopolysaccharide in marine bathing water; a potential
real-time biomarker of bacterial contamination and
relevance to human health**

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Abstract

The quality of marine bathing water is currently assessed by monitoring the levels of faecal indicator bacteria. Among other drawbacks, results are retrospective using the traditional culture based methods. A rapid method is thus needed as an early warning to bathers for bacterial contamination in marine bathing waters. Total lipopolysaccharide (LPS) was chosen here as a potential general biomarker for bacterial contamination. Levels of total LPS, measured using a Kinetic QCL™ Limulus Amebocyte Lysate (LAL) assay, highly correlated with enumerated *Escherichia coli* and *Bacteroides* species. Levels of LPS in excess of 50 EU mL⁻¹ were found to equate with water that was unsuitable for bathing under the current European Union regulations. Results showed that monitoring the levels of total LPS has a potential applicability as a rapid method for screening the quality of marine bathing water. More importantly, the LAL assay overcome the retrospective results when using culture based assessment since the LAL assay takes less than 30 minutes. Although false positive events were not detected, the occurrence of a false positive has been hypothesised, hence a more specific faecal biomarker was also investigated. LPS of five *Bacteroides* species (*B. fragilis*, *B. caccae*, *B. ovatus*, *B. xylanisolvens* and *B. finegoldii*) isolated from marine bathing waters samples were successfully profiled and showed high similarity between isolates in LPS gel electrophoresis banding pattern. Similar results were shown when investigating the endotoxic activity of *Bacteroides* species with the Kinetic QCL™ LAL assay. The potential biological relevance of *Bacteroides* LPS was also investigated in cell culture models indicating that *Bacteroides* showed similar induction of proinflammatory cytokines (TNF- α , IL-6 and IL-1 α) and generally the biological activity was approximately 100 fold less than *E. coli* LPS. In addition, an ELISA assay was designed for the detection of *Bacteroides* LPS. Results showed that the *Bacteroides* LPS has a high potential to be used as a faecal biomarker, however, further work is required to develop a fully functional assay. The potential biological relevance of LPS present in contaminated bathing waters was also investigated in cell culture models. Results showed that there is a significant difference in the production of proinflammatory cytokines in comparison to “clean” bathing waters. Thus, results suggest that the European Directive regulations should be extended to cover the levels of total LPS in bathing waters to assure safety to the users of marine recreational water.

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List of Abbreviations

APHA.....	American Public Health Association
<i>B. fragilis</i>	<i>Bacteroides fragilis</i>
BBE.....	<i>Bacteroides</i> Bile Esculin
Bcl10.....	B-Cell Lymphoma/Leukaemia 10
BLAST.....	Basic Local Alignment Search Tool
BPRM.....	<i>Bacteroides</i> Phage recovery Medium
BSA.....	Bovine Serum Albumin
BWR.....	Bathing Water Regulations
CD 14	Cluster of Differentiation 14
CFU.....	Colony Forming Unit
DEFRA.....	Department for Environment, Food and Rural Affairs
D. bathe.....	Deep bathing area
D. surf.....	Deep surfing area
DNA.....	Deoxyribonucleic Acid
dNTP.....	Deoxynucleotide Triphosphate
ddH ₂ O.....	Double distilled water
D.W.....	Distilled Water

EDTA.....	Ethylenediaminetetraacetic Acid
ELISA.....	Enzyme Linked Immunosorbent Assay
EU.....	Endotoxin Unit
FDA.....	Food and Drug Administration
GM-CSF.....	Granulocyte Macrophage Colony-Stimulating Factor
IKK α	I κ B Kinase Alpha
IKK β	I κ B Kinase Beta
IL-6.....	Interleukin Six
IL-1 α	Interleukin One Alpha
IL-8.....	Interleukin Eight
IMS.....	Industrial Methylated Spirits
IRAK1.....	Interleukin-1 Receptor-Associated Kinase 1
IRAK2.....	Interleukin-1 Receptor-Associated Kinase 2
IRAK4	Interleukin-1 Receptor-Associated Kinase 4
LAL.....	Limulus Amebocyte Lysate
LBP.....	LPS-Binding Protein
LPS.....	Lipopolysaccharide
Mal	MyD88 Adaptor-Like Protein

MALT1.....	Mucosa Associated Lymphoid Tissue 1
MD2	Myeloid Differentiation Factor 2
MDS.....	Multidimensional Scaling
mg.....	Milligram
MyD88.....	Myeloid Differentiation Primary Response 88
NEMO.....	NF-Kappa-B Essential Modulator Protein
NF- κ B.....	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
ng.....	Nanogram
nm.....	Nanometre
NSSP	National Shellfish Sanitation Program
NPDES.....	National Pollutant Discharge Elimination System
O.D.....	Optical Density
PCR.....	Polymerase Chain Reaction
PMB.....	Polymyxin B
qPCR.....	Quantitative Polymerase Chain Reaction
rRNA.....	Ribosomal Ribonucleic Acid
S. bathe... ..	Shallow Bathing Area
sCD 14.....	Soluble Cluster of Differentiation 14

SEPA.....	Scottish Environment Protection Agency
SDS-PAGE.....	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
S. surf.....	Shallow Surfing Area
TAB2	TAK1-Binding Protein 1
TAB3.....	TAK1-Binding Protein 3
TAE.....	Tris Acetate EDTA
TAK1.....	TGF-Beta Activated Kinase 1
TBS-T.....	Tris-Buffered Saline and Tween [®] 20
TE.....	Tris EDTA
TIR.....	Toll-interleukin-1 receptor
TIRAP	TIR-associated protein
TLR4.....	Toll-Like Receptor 4
TNA.....	Taq nuclease assay
TMB.....	3,3',5,5'-Tetramethylbenzidine
TNF- α	Tumour Necrosis Factor Alpha
TRAF6.....	TNF Receptor-Associated Factor 6
UNEP.....	United Nations Environment Program
UV.....	Ultraviolet

μmMicrometre

μLMicroliter

μgMicrogram

Presentations and Conferences

- Sattar, A., Bradley, G., and Jackson, S. (2013), "The potential of lipopolysaccharide as a real-time biomarker of bacterial contamination in marine bathing waters". *School of Biomedical and Biological Sciences seminar series, Plymouth University, UK, 05 June 2013 (oral presentation)*.
- Sattar, A., Habil, N., Jackson, S., Foey, A. and Bradley, G. (2012): "Endotoxin activity of *Bacteroides* species lipopolysaccharides from contaminated marine bathing water". *Proceedings of American Society for microbiology, San Francisco, USA (poster presentation) Page 181*.
- Sattar, A., Bradley, G. and Jackson, S. (2011), "Lipopolysaccharide as a real-time biomarker of human sewage pathogens in marine bathing waters". *Proceeding of the Society for applied Microbiology (SfAM) Summer Conference, Dublin 4-7 July (poster presentation,) Page 20*.
- Sattar, A., Bradley G., and Jackson S. (2011), "Lipopolysaccharide as a real time biomarker of human sewage pathogens in marine bathing waters". *Centre of Research in Translational Biomedicine (CRTB) research day, Plymouth University, 5th of April (poster presentation)*.
- Sattar, A., Bradley G., and Jackson S. (2011), "Lipopolysaccharide as a real-time biomarker of human sewage pathogens in marine bathing waters". *Postgraduate society conference series, Plymouth University, UK, 17th of March 2011 (best poster presentation prize)*.

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Peer Reviewed Publications

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Sattar A., Jackson S., Abate W., Fejer G. and Bradley G. (2014) proinflammatory effects of contaminated marine bathing water in *in vitro* cell culture models (*submitted*).

Sattar A., Jackson S. and Bradley G. (2014) Endotoxic activity of *Bacteroides* lipopolysaccharides isolated from contaminated marine bathing waters using LAL assay and cell culture models (*Manuscript in preparation*).

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Dedication

I would like to dedicate this Thesis to my family for their unconditional support, both emotionally and financially throughout my study. In particular, the patience and understanding shown by my Father, Mother and Siblings during my studying years is greatly appreciated.

Author Declaration

At no time during the registration for the degree of PhD has the author been registered for any other University award, without prior agreement of the Graduate Committee.

Word count of the main body of thesis without references: 41,176

Signed.....

Date.....

Chapter 1: General introduction and literature review

1.1 Background and general introduction

The use of recreational water is growing with easier transportation and warmer weather during the summer time (Pond, 2005). Water-related recreational activities possess several biological health hazards depending on various factors, such as type of health hazard, the quality of bathing water and immune susceptibility of bathers. Previous studies including outbreak reports have made links between health, illnesses and immersion in poor quality bathing waters difficult to attribute illnesses to bathing waters. To date, there are still unanswered questions concerning severity and occurrence of health hazards associated with bathing waters. It is conceivable that severe sickness may possibly result from bathing in recreational waters that have a high number of microorganisms; however, this association has only been investigated to a minor extent. Acute infections that are linked to bathing waters have been well documented (Pond, 2005); however, the consequences of these diseases need to be addressed.

The quality of marine bathing waters in Europe is legislated and governed by the European Directive (2006). The quality of marine bathing waters is assessed by enumerating the numbers of “bacterial indicators” in water samples collected from such waters using culture-based methods. These culture based methods have several drawbacks and results are retrospective taking 1-2 days; in addition the quality of beaches is determined based on results of previous years. qPCR methods have been considered for monitoring the quality of

bathing waters in addition to the source of microbial source tracking, however, these methods are expensive, require highly skilled personnel and are unsuitable for untrained bathing water users to conduct *in situ*. This research focused on the use of lipopolysaccharide (LPS) as a real-time biomarker for quality of bathing water quality monitoring. The biological relevance of contaminated bathing waters has only been explored by surveying bathing water users after being in contact with the beach. Because there is no direct link between the status of bathing water quality and human health, cell culture models were developed here to investigate the potential biological relevance of contaminated bathing waters to human health.

1.2 Marine bathing waters and potential human health risks

Bathing as defined in the dictionary (Stevenson, 2010) refers to the immersion of the body in fluid, and bathing waters are defined as fresh or seawater in which bathing is authorised and is traditionally practised by a large number of bathers and other water sports performers (SEPA, 2002). Bathing seawater becomes polluted when harmful substances are disposed altering the uniquely balanced marine ecosystem, and may cause harmful effects to creatures in contact with the pollutants directly (marine creatures) or indirectly (Human, seagull and other birds). Harmful pollutants may include spillage of oil from oil tankers and different types of waste (chemical, agricultural, industrial). Different sources of faecal contamination in marine bathing waters have been identified and are summarised in figure 1.1.

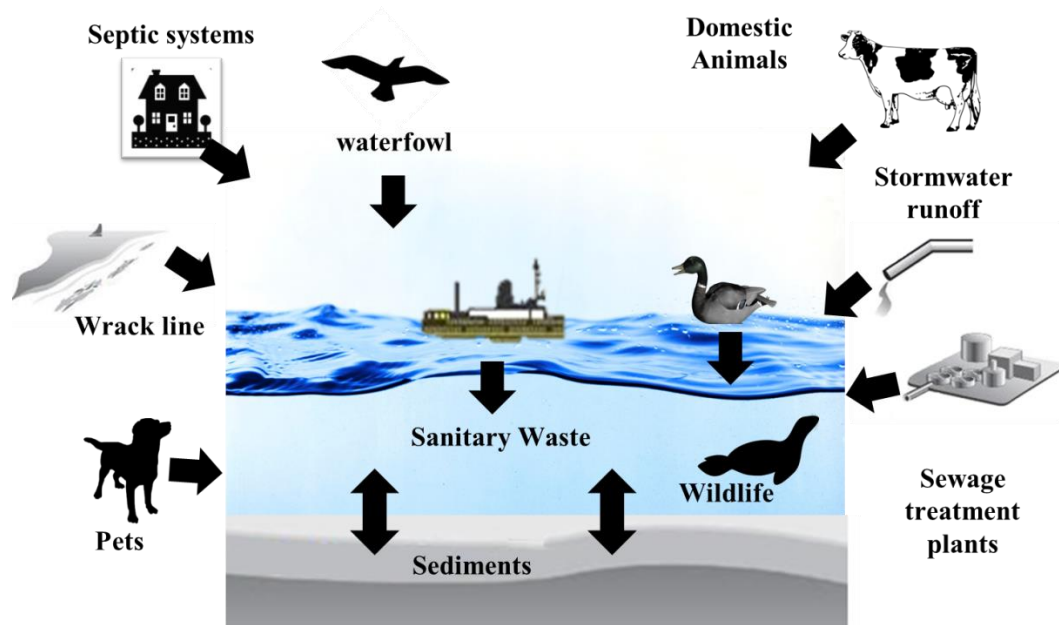


Figure 1.1: Potential sources of bacterial contamination in an estuary, redrawn from Ely, 1997. Cited in Ohrel and Register (2002).

Bacterial and viral pathogens enter a body of water via the contamination with faecal material as a result of poorly treated sewage (primary or secondary treatment of sewage in less developed countries), damaged or leaky septic systems, runoff from urban areas, boat, yacht and marina waste products, combined sewer overflows, pet waste, farm animals and wildlife (NPDES, 2006). Human sickness can result from drinking polluted water or swimming/bathing in water that contains pathogens, or from eating shellfish harvested from such waters.

When bathers perform water-related activities they are in constant contact with microorganisms that are either endogenous or exogenous that might present from different sources especially faecal contamination sources. Several studies strongly suggest that human illness increase when being in contact with polluted bathing waters with high number of faecal bacterial indicators (Cabelli

et al., 1979, Cabelli *et al.*, 1982, Cabelli, 1983, Cabelli *et al.*, 1983, Cabelli, 1989, Balarajan *et al.*, 1991, Prieto *et al.*, 2001). Bacterially and virally polluted bathing waters have a potential risk to human health, by humans being in contact directly with contaminated waters when bathing or indirectly when fishing or shellfish harvesting for food (Scoging, 1991). These can include a variety of viral and bacterial infections, for example: hepatitis and peritonitis (Gleick, 2002, Isogai *et al.*, 1989). The quality of marine bathing waters is investigated by enumeration of faecal indicator bacteria (FIB) because it is expensive and time consuming to investigate the presence of pathogens in marine bathing waters. Traditionally, FIB have been used to suggest the presence of pathogens (Berg 1978) including bacteria, protozoa and viruses. Some potential pathogens present in bathing water are listed in table 1.1.

Table 1.1 Some Common pathogens present in contaminated marine and fresh bathing waters (Ashbolt, 2004).

<i>Adenoviridae virus</i>	<i>Legionella pneumophila</i>	<i>Vibrio cholerae</i>
<i>Entamoeba histolytica</i>	<i>Salmonella</i>	<i>Escherichia coli</i>
<i>Campylobacter jejuni</i>	<i>Coronavirus</i>	<i>Giardia lamblia</i>
<i>Cryptosporidium</i>	<i>Calicivirus</i>	Hepatitis A virus

The quality of marine bathing waters in the UK is governed by the European Union bathing water directive. In England and Wales, the Department for Environment, Food & Rural Affairs (DEFRA is the governmental department responsible to preserve, protect, improving and enforce testing of designated

marine bathing waters for harmful substances for bathers, swimmers, surfers, fishermen and any other humans who are in contact with bathing waters. Testing of designated marine bathing water includes is carried out by monitoring the levels of FIB. Bathing water quality is monitored on a regular basis during the bathing season in Wales and England from 15th of May to 30th of September (BWR, 2008).

1.3 Faecal indicator bacteria (FIB)

Pathogenic microorganisms can be found in marine ecosystems and it is difficult and expensive to directly monitor and test bathing sea water for all these pathogenic microorganisms (Rhodes and and Kator, 1991). Instead, monitoring is done using a certain bacterium or a group of bacteria as indicators for these pathogens. Thus, bathing water is monitored in terms of microbiological pollution using faecal indicator bacteria (Godfree *et al.*, 1997). The concept of “Bacterial indicators” of the sanitary quality of water most likely dates back to 1880 when Von Fritsch described *Klebsiella pneumoniae* and *Klebsiella rhinoscleromatis* as microorganisms habitually found in human faeces (Geldreich, 1970) that indicate the possible presence of pathogenic microorganisms. Different bacteria were chosen over the years, then replaced in order to choose the best representative and cost effective bacterial indicator. However, even until the present day, there is no such thing as the “ideal” or the “universal indicator” for a variety of reasons. On the other hand, since no organism can be a universal indicator, a good bacterial indicator organism

should meet as many of the following criteria as possible (Markhali, 2009 , Godfree *et al.*, 1997). Hence, bacterial indicators should be:

1. Suitable for the analysis of all kinds of water: potable, river, ground, impounded recreational, estuary, sea, and waste.
2. Present whenever pathogens are present.
3. Able to survive longer than the hardiest pathogens.
4. Unable to reproduce in the contaminated water.
5. Able to show a high specificity; i.e. other bacteria should not give positive results.
6. Detected with an easy to perform testing method.
7. Illness risk-free to humans.
8. Reflect the degree of faecal pollution.

Previous and current faecal bacterial indicators and their detection methods are listed below:

1. A total coliform test is a standard test to investigate the quality of bathing waters by detecting the presence of coliform bacteria. Coliforms are bacteria that are present in the digestive tracts of humans and animals and are found in their wastes or within the environment. Coliforms are rod-shaped, non spore-forming, lactose fermenting at 37 °C (Gillespie

and Hawkey, 2006), facultative anaerobic Gram-negative bacteria and were used as bacterial indicators for bathing waters until replaced because of being non-specific indicators of faecal pollution. Total coliforms include: *Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter amnigenus* and *Citrobacter freundii* (Alonso *et al.*, 1996). Since total coliforms include species present in the environment, results may indicate non accurate representation (UNEP/WHO, 1983).

Total coliform test can be performed in the following two ways:

A- Membrane Filtration method (APHA., 1995.).

B- Most probable number method (MPN) (APHA., 1995.) .

2. Faecal coliforms: are so called due to their role as faecal indicators. Faecal coliforms ferment lactose and produce acid and gas at 44.5 ± 0.2 °C within 24 hours. Faecal coliform bacteria are found in the faeces of human and other warm-blooded animals as described in several studies e.g. (Gerba, 2000, Leeming *et al.*, 1996). These bacteria can enter rivers from agricultural and storm runoff carrying wastes from birds such as seagulls and mammals such as livestock, and from human sewage discharged into the water. Faecal coliforms are commonly used to test recreational waters and are approved as an indicator for water quality by the U.S. Food and Drug Administration's National Shellfish Sanitation Program (NSSP) for classifying shellfishing waters. On the other hand, this group also includes a few species that can be non-faecal in origin such as *Klebsiella pneumoniae*, which grows well in paper pulp and sometimes found in high concentration near paper factories (Gauthier *et al.*, 2000). Studies have found that all members of the coliform group can re-grow in natural surface water depending on water temperature and amount of organic matter

(Gleeson and Gray, 1997) cited in (Gerba, 2000, Raina M. Maier *et al.*, 2009).

Faecal coliform test can be performed in the following two ways:

A- Membrane filtration method (HMSO., 1982).

B- Multiple Test Tubes: (APHA, 1995).

3. Faecal streptococci: Gram-positive, catalase-negative cocci that grow on bile esculin agar at 45°C, belonging to the genera *Enterococcus* and *Streptococcus* possessing the Lancefield group D antigen (Hardie and Whiley, 1995).
4. *E. coli*: straight bacilli, facultative anaerobic, Gram-negative bacteria that normally inhabit the lower intestine of human and animals as part of normal gut flora. *E. coli* is a member of the *Enterobacteriaceae* that can be distinguished from most other coliforms by their capability to ferment lactose at 44 °C.
5. Intestinal enterococci: are Gram-positive, facultative anaerobic (Fischetti and Microbiology, 2000) subset of faecal streptococci that grow at pH 9.6, 45 °C in 6.5% NaCl, resistant to 60 °C for 30 minutes and able to reduce 0.1% methylene blue. Enterococci are frequently found in pairs (diplococci) or in short chains and are hard to differentiate from streptococci using physical characteristics alone. Faecal streptococci were substituted by intestinal enterococci in 2006 as stated in the European Union water Directive (2006).

Generally, increased levels of faecal coliforms in marine bathing waters provide a warning of failure in water treatment, a break in the septic system and

potential contamination with pathogens. Currently, culture based methods are used to investigate the quality of marine bathing waters by filtering 100 mL of marine bathing water through a 0.45 μm membrane and enumerating the current bacterial indicators: *E. coli* and intestinal enterococci (European Directive, 2006). The European Directive has classified the quality of bathing waters into Excellent, Good, Sufficient and Poor depending on the number of enumerated bacterial indicators. Table 1.2 shows the values of each water quality rank.

Table 1.2: bathing water quality ranks for coastal waters set by the European Bathing Water Directive 2006/7/EC

	Parameter	Excellent	Good	Sufficient
1	Intestinal enterococci (CFU100 mL ⁻¹)	100 (*)	200 (*)	185 (**)
2	<i>Escherichia coli</i> (CFU100 mL ⁻¹)	250 (*)	500 (*)	500 (**)
(*) Based upon a 95-percentile evaluation.				
(**) Based upon a 90-percentile evaluation.				
Poor quality (Fail): the number of bacterial indicators greater than the 'sufficient' rank values.				

However, studies have shown that faecal indicator bacteria have several drawbacks, including growth of bacteria in seawater in addition to results being retrospective, taking 24-48 hours, since the current method in use to evaluate the quality of bathing water is culture based (Anderson *et al.*, 2005, Borrego J.J. *et al.*, 1983, Field and Samadpour, 2007).

1.4 Polymerase Chain Reaction (PCR) methods for monitoring microbial source tracking (MST)

Microbial source tracking is a set of methods conducted to investigate the source that contributes faecal pollution to marine bathing waters where sewage and faecal contamination is common in such waters, since standard culture-based membrane filtration method used to measure faecal contamination in recreational water provide no evidence of the contamination source. *Bacteroides*-Host specific MST method is used to mainly identify if human faecal contamination source is present. Knowing the source of faecal contamination is a key role in the process of treatment and elimination of the faecal source as it can cause health risks for humans. Faecal pollutions are classified as point and non-point sources. Point sources include sewage and effluent from wastewater treatment plants while non-point sources may include storm water, leakage from septic systems, agriculture or wildlife runoffs where the source of faecal contamination to bathing waters is ambiguous. Storm events appear to be the best example for non-point source of faecal contamination which perhaps is the main cause of water quality deterioration (Hilgenkamp, 2005). Compromised sewage or septic systems might be the culprit when human specific biomarkers appear positive. Although it is important to identify the source of faecal contamination of animals (dog, cow, etc...) it is more important to investigate the presence of human faecal contamination. The presence of human faecal biomarker implies that there is a high risk of human pathogen passed through faecal material and could be transmitted into another human through the oral cavity (faecal-oral route). This will increase the

transmission of a variety of diseases such as typhoid fever, cholera, giardiasis, Hepatitis A virus and Norwalk virus outbreaks etc. (Ashbolt, 2004). Four methods have been established to investigate the source of faecal contamination, namely phenotypic, microbiological, chemical and genotypic methods (Scott *et al.*, 2002). All four methods have advantages and disadvantages; however, no single method can replace the other three. The main advantage of genotypic methods is the discrimination of human from any other faecal contamination using host-specific biomarkers. Highly conserved *Bacteroides* host-specific molecular markers that likely reflect differences in host animal digestive systems have been targeted using PCR-based methods by designing primers specific to amplify these markers. These primer sets have been described, and successfully tested in the past two decades showing high specificity and sensitivity to identify and discriminate different faecal contamination sources such as human, cow, sheep, birds and other hosts (Okabe and Shimazu, 2007, Bernhard and Field, 2000a, Bernhard and Field, 2000b).

A rapid quantitative polymerase chain reaction (qPCR) method for faecal *Bacteroides* has been developed which has the potential to be a tool for assessing the source of faecal contamination in bathing waters (Converse *et al.*, 2009). This method is based on the recommendation of several previous studies which suggested that *Bacteroides* species represent an excellent potential bacterial indicator. This method uses a PCR assay targeting the 16S rRNA gene of *Bacteroides* species present in human intestine. According to Converse (2009), this method might be a helpful assay to efficiently predict the

occurrence of human faecal contamination. Other studies have targeted *E. coli* and enterococci 16S rRNA genes as a rapid method to assess the quality of marine bathing waters (Bergeron *et al.*, 2011, Bushon *et al.*, 2009). However, these methods are expensive, complex in requiring skilful personnel and inconvenient to use on site.

1.4.1 *Bacteroides* Species

Bacteroides species are anaerobic Gram-negative bacilli with rounded ends about 0.5 to 0.8 µm in diameter and 1.5 to 4.5 µm long (Baron, 1996), and are chemo-organotroph (organisms which use organic compounds as their energy source). *Bacteroides* species are pleomorphic rod shaped bacteria that are predominant components of the normal flora of the mucosal membrane in the human intestine, they are even more predominant than *E. coli* (Fiksdal *et al.*, 1985). Hence, *Bacteroides* can give a more accurate faecal contamination indication than *E. coli* since *Bacteroides* species are only present in the faeces of human and warm blooded animals (Kreader, 1995). *Bacteroides* species are non-endospore-forming and may be either motile or non-motile, depending on the species with a guanine-plus-cytosine content of 42% (Norwalk and Appleton, 1995). Certain species of *Bacteroides*, for example *B. fragilis*, are opportunistic pathogens and can cause abscesses in the abdomen, cranium, thorax, peritoneum, liver, appendicitis and female genital tract; these localised abscesses are proven to be caused by *Bacteroides* species after being isolated from the abscess sites (Ryan, 2004). In some cases, it has been found that patients with malignant diseases suffered from septicaemia caused by *Bacteroides* species and eight patients died because of the opportunistic

Bacteroides (Joseph and Julian, 1970). On the other hand, *Bacteroides* species play an important role as the normal flora bacteria which compete with other potentially pathogenic bacterial species and prevent them from colonising the intestinal tract (Norwalk and Appleton, 1995). *Bacteroides* are fastidious anaerobic species; for this reason, they are difficult to isolate and often overlooked (Jousime-Somers *et al.*, 2002). When *Bacteroides* species are isolated on *Bacteroides* Bile Esculin (BBE) agar, a brown or black colour surrounds colonies because of esculin hydrolysis. Some strains of *B. vulgatus*, a member of *B. fragilis* group, can test esculin-negative. *Bacteroides* are classified as the following:

Kingdom: Bacteria, Phylum: Bacteroidetes, Class: Bacteroidetes, Order: Bacteroidales, Family: Bacteroidaceae, Genus *Bacteroides*. Table 1.3 lists *Bacteroides* species.

Table 1.3: Species of the genus *Bacteroides* (Euzéby, 2013)

<i>B. castellani</i>	<i>B. acidifaciens</i>	<i>B. barnesiae</i>	<i>B. caccae</i>	<i>B. rodentium</i>
<i>B. cellulosilyticus</i>	<i>B. cellulosolvens</i>	<i>B. clarus</i>	<i>B. coagulans</i>	<i>B. salanitronis</i>
<i>B. paurosaccharolyticus</i>	<i>B. ovatus</i>	<i>B. pectinophilus</i>	<i>B. polypragmatus</i>	<i>B. salyersiae</i>
<i>B. oleiciplenus</i>	<i>B. nordii</i>	<i>B. plebeius</i>	<i>B. propionificaciens</i>	<i>B. sartorii</i>
<i>B. xylanisolvens</i>	<i>B. gallinarum</i>	<i>B. graminisolvens</i>	<i>B. intestinalis</i>	<i>B. stercorisoris</i>
<i>B. fragilis</i>	<i>B. vulgatus</i>	<i>B. galacturonicus</i>	<i>B. helcogenes</i>	<i>B. xylanolyticus</i>
<i>B. coprocola</i>	<i>B. faecichinchillae</i>	<i>B. faecis</i>	<i>B. finegoldii</i>	<i>B. stercoris</i>
<i>B. coprophilus</i>	<i>B. coprosuis</i>	<i>B. dorei</i>	<i>B. eggerthii</i>	<i>B. tectus</i>
<i>B. thetaiotaomicron</i>				

1.4.2 *Bacteroides* as a potential bacterial indicator

Several studies have suggested the use of *Bacteroides* species as alternative to faecal indicator organisms, because they are strictly present in the intestine of human and warm-blooded animals and constitute a substantial portion of the faecal bacterial population (Madigan *et al.*, 2000). In addition, *Bacteroides* have a highly conserved host specificity that mirrors variances in the digestive system of different hosts (Bernhard and Field, 2000b). *Bacteroides* species are also unable to grow in the environment, mainly because of their anaerobic nature and high demand nutritious factors. Therefore, *Bacteroides* species can indicate a recent faecal contamination. Furthermore, *Bacteroides fragilis* represent a suitable host for *Bacteroides* bacteriophage and these phages can be indicators for enteric viruses. Studies conducted by (Fiksdal *et al.*, 1985, Allsop and Stickler, 1985, Kreader, 1995) support and recommend using *Bacteroides* species as bacterial indicators. Table 1.4 shows the most recent studies supporting the use of *Bacteroides* as a faecal indicator in water:

Table1.4: Recent studies showing the importance of *Bacteroides* Species as bathing water indicators.

Reference	Type or purpose of experiment	Sample type	Methods used	Results /Recommendations
(Kreader, 1995)	PCR primers specific to the 16S rRNA gene sequences of <i>Bacteroides distasonis</i> , <i>B. thetaiotaomicron</i> , and <i>B. vulgatus</i> were designed	Faeces (human and non-human)	PCR	Further studies to show whether these assays can be used to distinguish sewage from farm runoff was suggested.
(Bernhard and Field, 2000b)	Develop an inexpensive, rapid method of diagnosing the source of faecal pollution in water	Water samples	PCR	Designing a specific PCR primers that discriminate human and ruminant sources of faecal contamination
(Dick and Field, 2004)	Quantitative Taq nuclease assay for faecal pollution using a <i>Bacteroidetes</i> 16S rDNA marker	Seawater samples	PCR	TNA for 16S rDNA of <i>Bacteroidetes</i> is rapid, sensitive, and reproducible in sewage dilutions.
(Carson <i>et al.</i> , 2005)	Test the specificity of a <i>Bacteroides thetaiotaomicron</i> as a marker for Human faeces	Faecal samples (human and animals)	PCR	The marker was not detected in 1 ng. of faecal DNA from cows, horses, pigs, chickens, turkeys, and geese

Table1.4 (continued) : Recent studies showing the importance of *Bacteroides* Species as bathing water indicators

(Layton <i>et al.</i> , 2006)	Design a real-time PCR assay to target <i>Bacteroides</i> species (AllBac) present in human, cattle, and horses faeces.	Faecal samples (human and animals)	Real-Time PCR	AllBac assay provides a rapid direct measurement of faecal contamination in water and may complement <i>E. coli</i> as a faecal indicator.
(Kildare <i>et al.</i> , 2007)	Design a 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific faecal Bacteroidales	Faecal samples (human and animals)	Real-time TaqMan PCR	Identification of a new universal marker sequence for the quantitative detection of faecal Bacteroidales
(Reischer <i>et al.</i> , 2007)	Developing of a sensitive human-specific quantitative real-time PCR assay for microbial faecal source tracking	Faecal and waste water samples	Real-time PCR	Adequate sensitivity allowing quantitative source tracking of human faecal impact in the investigated water body.
(Ballesté and Blanch, 2010)	Survival of <i>Bacteroides</i> species in the environment	River water samples	Culture based, PCR and qPCR	<i>Bacteroides</i> can be used as markers of recent faecal pollution. Environmental <i>Bacteroides</i> strains survived longer than either type strain, due to better adaptation to environmental conditions

Table1.4 (continued) : Recent studies showing the importance of *Bacteroides* Species as bathing water indicator

(Liang <i>et al.</i> , 2012)	Persistence of <i>Bacteroides</i> species in freshwater	freshwater microcosms spiked with human/bovine faeces	qPCR, DGGE	Both human and cow host specific <i>Bacteroides</i> biomarkers decayed significantly faster than <i>E. coli</i>
(Tambalo <i>et al.</i> , 2012)	assessment of two qPCR methods targeting a dog-related <i>Bacteroidales</i> 16S rRNA biomarker	Human, pig, horse, deer, mountain goat, bison, caribou, and moose faecal and environmental samples	qPCR	Results promote the use of the dog mtDNA assay in detecting dog faecal contamination in water samples.
(Liang <i>et al.</i> , 2013)	Investigate the association of FIB, <i>Salmonella</i> and <i>Bacteroides</i> human and cow biomarkers with the water physico-chemical parameters	Water samples from 12 surface runoff	qPCR	Water temperature, dissolved organic carbon (DOC), and nutrient levels were positively correlated with FIB concentrations.

1.5 The use of total LPS as a rapid biomarker for bacterial contamination

Several studies have attempted to use total LPS as an indicator of bacterial contamination in different waters such as industrial plant effluent, potable and reclaimed water, however, none have actually used it as a biomarker in marine bathing waters. Table 1.5 summarise studies conducted using LPS as an indicator of bacterial contamination.

Table 1.5: Summary of studies conducted using LPS as an indicator of bacterial contamination

References	Type or purpose of experiment	Sample type	Methods used	Results /recommendations
(Jorgensen <i>et al.</i> , 1973)	Detection of endotoxins of Gram-negative bacteriuria in children	Urine	Clot method	Easy to use with high correlation to culture based methods. rapid presumptive detection of Gram-negative bacteriuria in patients
(Jorgensen <i>et al.</i> , 1976)	Detection of endotoxins in potable waters and from reclaimed advanced treatment plant effluent	Plant effluent and potable waters	Clot method	Easy to use, chlorination of water interferes with the assay.
(Watson <i>et al.</i> , 1977)	Estimation of bacterial numbers and biomass using epifluorescence microscope, transmission electron microscope and LAL assay	Seawater or bacterial cultures	Spectrophotometric LAL assay	The biomass of Gram-negative bacteria was shown to be related to the LPS in the samples. A factor of 6.35 was determined for converting LPS to bacterial carbon.

Table 1.5 (continued): Summary of studies conducted using LPS as an indicator of bacterial contamination

References	Type or purpose of experiment	Sample type	Methods used	Results /recommendations
(Jorgensen <i>et al.</i> , 1979)	Comparison of LAL assay to standard plate count and total coliform count	Reclaimed water	Clot method	Endotoxin values did not correlate extremely well with determinations of viable bacterial numbers
(Haas <i>et al.</i> , 1983)	Endotoxin assay of Gram-negative bacteria correlated to standard plate and total coliforms	Water treatment and distribution systems	Photometric LAL assay	Relatively low level of association between water microbiology and endotoxins

1.5.1 General structure of lipopolysaccharide

Richard Friedrich Johannes Pfeiffer, (1858-1945) was a German bacteriologist and immunologist who first discovered lipopolysaccharide and planted the seed for the subsequent work involving this potent molecule until the present day. The term lipopolysaccharide (LPS), which is interchangeably used with endotoxin throughout this thesis, is a complex molecule consist of lipid and polysaccharide moieties secured to the outer membrane by ionic and hydrophobic forces, and its strong negative charge is neutralized by the two ions Ca^{2+} and Mg^{2+} (Rietschel *et al.*, 1994, Raetz, 1990). The main function of the lipopolysaccharide is to support the bacterial structure and to protect it from the exterior factors such as lysozymes and other antimicrobial agents that might affect the bacterial cell. Lipopolysaccharide structures have gained, over the last century, a vast attention by researchers endeavouring to understand their role, potency and health effects on human health. In general, lipopolysaccharide is composed of three regions (figure 1.2):

1. O antigen: also known as O-specific chain is a hydrophilic repetitive polysaccharide moiety of LPS. The O antigen is attached to the core oligosaccharide. The O antigen is a highly variable region since different bacteria possess different repeating numbers of the polysaccharide (Raetz and Whitfield, 2002).
2. Core oligosaccharide: also known as core region can be divided into inner and an outer subdomain. Core oligosaccharide is a short chain of sugars that link lipid A to the O- antigen (Raetz, 1990).

3. Lipid A: a hydrophobic moiety anchoring the LPS molecule in the Gram-negative bacterium outer membrane. Lipid A consists of two glucosamine units with attached acyl chains containing usually one phosphate group on each glucosamine (Raetz *et al.*, 2009).

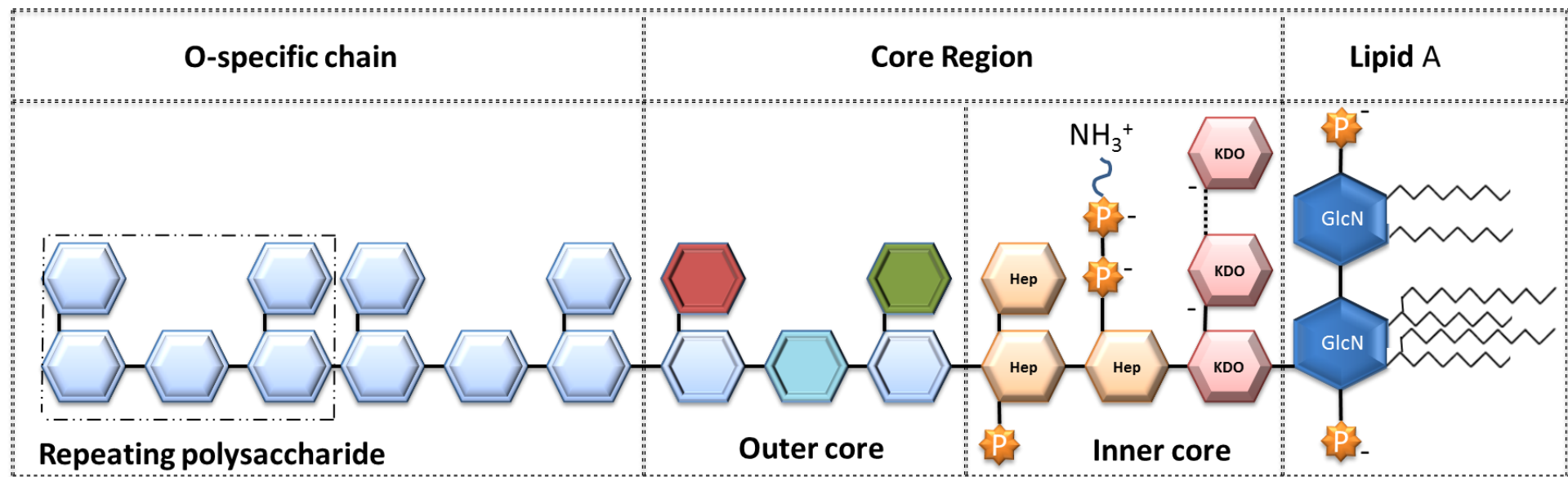


Figure 1.2: A generic chemical structure of lipopolysaccharide from Gram-negative bacteria consists of Lipid A; a membrane anchored phospholipid consist of 6 acyl groups and 2 phosphate groups. Numbers of acyl and phosphate groups might vary from on bacterial species to another. Lipid A region is covalently linked to the core region. Smooth form LPS has O-specific chain and a repeating sugar unit which gives the ladder-like shape when LPS separated in SDS PAGE. GlcN, glucosamine; Kdo, '2-keto-3-deoxyoctulosonic acid' (3-deoxy-D-manno-octulosonic acid); Hep, D-glycero-D-manno-heptose. Adapted and modified from Alexander and Rietschel (2001).

1.5.2 Principle of Limulus Amebocyte Assay

The Limulus amebocyte lysate (LAL) assay is extremely sensitive to LPS used to quantify the activity of LPS of Gram-negative bacteria. Bang (1956) noticed that Gram-negative bacteria (*Vibrio* species) coagulate the plasma of the horseshoe crab *Limulus polyphemus* and eventually cause death. Bang and Levin linked the coagulation of horseshoe crab plasma to the presence of lipopolysaccharide in Gram-negative bacteria which led later to the production of a lysate of the amebocyte from the *Limulus polyphemus* horseshoe crab (Bang, 1956, Levin and Bang, 1964). Later, it was discovered that the cause of this intravascular coagulation was enzymatic (Young *et al.*, 1972, Solum, 1970, Solum, 1973). The LAL assay has been approved by the FDA as a standard method to test for bacterial LPS contamination in pharmaceutical industries. There are three methods to detect and quantify LPS in products, the LAL gel clot method, Kinetic turbidimetric LAL assay method, and the chromogenic assay.

The enzymatic cascade of the gel clot LAL assay uses an LPS triggered enzyme cascade from horseshoe crab lysate. Initially, factor C is activated by LPS, then the activated factor C activates factor B, active factor B activates a proclotting enzyme into clotting enzyme that finally lead to initiate coagulation. The Kinetic turbidimetric assay uses the same principle of the gel clot LAL assay method but the reaction is automatically monitored over time for the appearance of turbidity.

This method was later developed to become a chromogenic method and eventually to Kinetic QCL™ LAL assay. The Kinetic QCL™ LAL assay also uses the same principle of factor C activation cascade, however, instead of a coagulation reaction as an end product, an enzyme cleaves the colourless synthetic substrate (Ac-Ile-Glu-Ala-Arg-pNA) producing a yellow colour (p-nitroaniline) (figure 1.3).

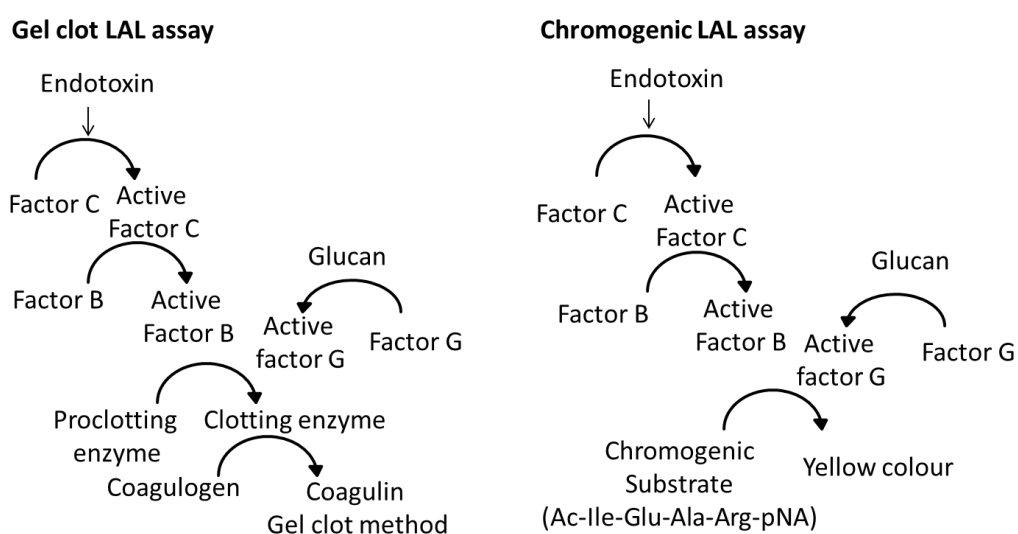


Figure 1.3: A schematic diagram of gel clot and chromogenic Limulus Amebocyte Lysate (LAL) assay.

The activity of LPS is expressed in Endotoxin Units per mL of sample (EU mL^{-1}). The food and drug agency (FDA, USA) defined the endotoxin unit (EU) as the endotoxin activity of 0.2 ng of reference endotoxin standard depending on the source of endotoxin (Liebers *et al.*, 2006)..

1.5.3 Relationship of structure and biological activity of LPS

LPS and particularly Lipid A moiety is by far the most potent molecule in the bacterial cell wall in regard to triggering an immunological reaction in mammalian macrophages, monocytes and dendritic cells. (Rietschel *et al.*, 1996, Holst *et al.*, 1996). Researchers have discovered that unique structural organisation and conformation of lipid A defines the endotoxic activity (potency) of LPS (Rietschel *et al.*, 1993). Partial structures of lipid A were chemically synthesised and tested to determine the factor by which the synthesised structure was less potent than the original lipid A moiety (Rietschel *et al.*, 1994). For example, the potency of LPS is decreased by a factor of 10^2 in the absence of one of the phosphate groups that is attached to the glucosamine molecules. Similarly, the biological activity of LPS decreases when the number of acyl groups decrease. A schematic structure of lipid A and the factor of activity decrease are illustrated in figure 1.4.

1.5.4 *Bacteroides* LPS as a potential faecal-specific bacterial indicator

Studies have shown that LPS of *B. fragilis* contains little 2-ketodeoxyoctanate, or heptose and lacks or has a little O-specific chain, hence the LPS of *Bacteroides* species is considered as rough LPS (Baron, 1996, Rietschel *et al.*, 1993). This LPS exhibits little endotoxic activity in LAL assay and little chemotactic activity to attract leucocytes in the human body (Yoshimura *et al.*,

1987). A study conducted by Weintraub *et al* (1989) has shown that the lipid A of *Bacteroides fragilis* contains only one ester-bound phosphate and only five acyl residues. These unique features of *Bacteroides fragilis* lipid A differentiate it from other lipid A from different genera of Gram-negative bacteria (figure 1.4). In 1990 (Lindberg *et al.*) researchers have found out that the endotoxic activity of extracted LPS from *Bacteroides fragilis* is 100-1000 fold less than *E. coli* and *Salmonella* in stimulation the local Schwartzman reaction and production of IL-1 α . Rietschel *et al* (1994) have demonstrated that that low number of acylation and phosphorylation of lipid A in *Bacteroides fragilis* is responsible for the poor biological activity (figure 1.4). Likewise, low biological activity of LPS has been confirmed for the closely related species *B. thetaiotaomicron*, and *B. ovatus* (Baron, 1996).

There are no previous studies that suggest the use of LPS from *Bacteroides* species as a potential biomarker for faecal contamination in marine bathing waters. However, special attention was paid to the physical structure and banding pattern of *Bacteroides* LPS from clinical samples showing that the LPS of most *Bacteroides* species consisted of rough LPS and a few series of high molecular weight bands (Maskell, 1991, Maskell, 1994). However, *Bacteroides* species have been under major reclassification since then, and a few of the *Bacteroides* species have been reclassified into entirely different genera (Sakamoto and Benno, 2006). In addition, it has been proven that the different methods used in the extraction of LPS can yield different chemical composition and activity (Poxton and Edmond, 1995).

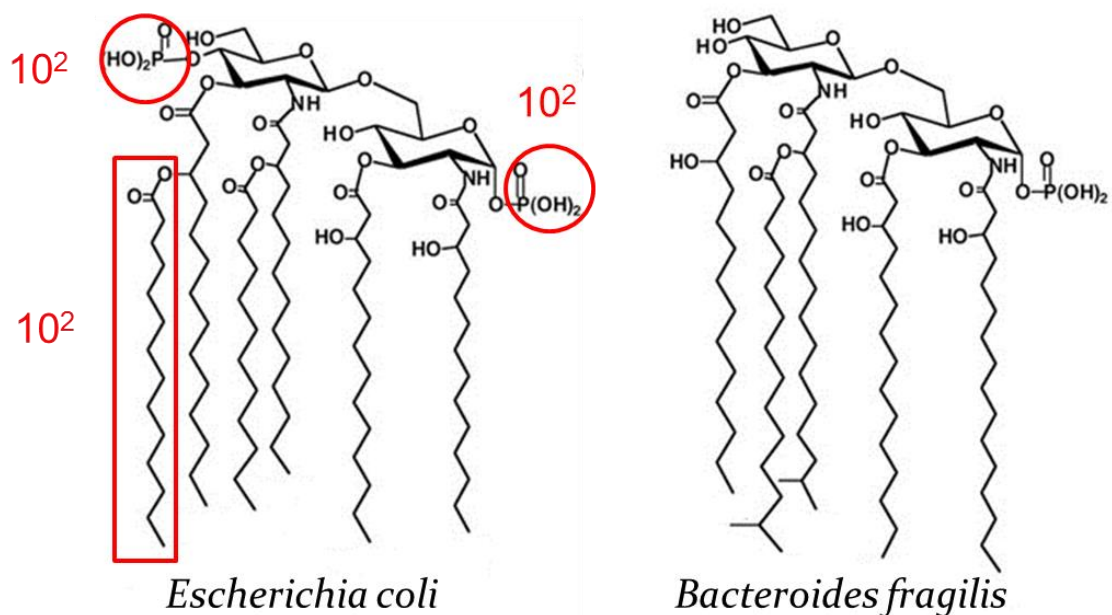


Figure 1.4: Chemical structure of lipid A. of *Escherichia coli* and *Bacteroides fragilis*. Changes in the chemical structure of lipid A of *Escherichia coli* (red circles for phosphate and red rectangle for acyl group) and the approximate fold decrease of biological activity is also illustrated. Adapted and modified from (Weintraub *et al.*, 1989) and (Rietschel *et al.*, 1993).

1.6 Seawater bathing and human immunity

The immune system of humans is in a daily confrontation with various challenges. A human becomes in close contact with indigenous and possibly pathogenic microbes when bathing in poor quality. The innate immune system represents the first line of host defence during infection and thus plays an important role in the initial recognition followed by triggering of immunological mediators such as cytokines and chemokines. On the other hand, the adaptive immune system is responsible for eradication of pathogenic microbes in the late stage of infection (Owen *et al.*, 2013). Pattern recognition receptors (PRR) are receptors present on the cell wall or in the cytosol of most cell types that can recognise pathogen-associated molecular patterns (PAMPs) such as

monocytes, macrophages and dendritic cells. PAMPs have an evolutionary conserved patterns in pathogens and can be recognised by the host immune system as a foreign body (Kim *et al.*, 2007). As part of the innate immune system, Toll-like receptors (TLRs) are type of PRR trans-membrane proteins that were first discovered in *Drosophila* flies (Nüsslein-Volhard and Wieschaus, 1980). Ten different TLRs have been identified in humans which recognise different molecules of different PAMPs (Janssens and Beyaert, 2003). It is known that LPS signals through TLR 4, is one of the most potent molecules that trigger an overwhelming immune response that could lead to serious health conditions and even death (Marzocco *et al.*, 2004, Yamamoto *et al.*, 2011).

1.6.1 The use of cell culture models in determining health relevance (risk) of contaminated bathing waters.

The potential biological relevance (risk) of contaminated bathing waters is usually assessed by epidemiological-microbiological studies. These studies were surveys linking the microbial indicators to health effects at marine bathing beaches. These studies showed that there is a correlation between the levels of FIB and human health problems such as gastroenteritis and infection of respiratory systems (Cabelli, 1983, Cabelli, 1989, Cabelli *et al.*, 1979, Cabelli *et al.*, 1982, Cabelli *et al.*, 1983). However, it is plausible that bathers could become ill virtually from any contaminant such as consuming contaminated sandwiches, ice cream, etc. that is difficult to be linked to poor quality of bathing water. In addition, there are several factors determining the severity of an infection including the duration of water activity, type of water activity (Bradley

and Hancock, 2003) and the immune status of bathers. A study conducted in the UK showed that gastroenteritis infections were considerably higher in persons exposed to bathing waters meeting the “sufficient” water quality level than in control people who were not in contact with the water (Kay *et al.*, 1994). Respiratory tract, ear and wound infections could similarly occur following bathing (Fleisher *et al.*, 1996; Oliver, 2005). These infectious agents perhaps are due to LPS of exogenous or endogenous bacteria present in the marine bathing water, mainly certain species of *Vibrio* which cause health illness and septicemia by direct contact as well as following consumption of contaminated shellfish. The European directive has no legislation regarding safe bathing with high levels of LPS which creates another drawback in the current culture based bathing water quality methods. Cell culture models have been used in previous studies to investigate the potential biological relevance (health risks) to various harmful substances exposure. The effect of fly ash aerosol on lung tissue was investigated using alveolar macrophages and epithelial cells and the levels of proinflammatory mediators was measured (Diabaté *et al.*, 2002). Cell culture models have been used to evaluate the inflammatory effects of airborne LPS emitted from composting sites in the UK (Deacon *et al.*, 2009, Liu *et al.*, 2011). These studies have used cell lines relevant to human health risks by measuring the levels of proinflammatory cytokines. However, no studies have been conducted to assess the potential biological relevance of contaminated marine bathing water and especially LPS in similar cell culture models.

1.6.2 TLR4 and LPS recognition

TLR4 is a type 1 transmembrane protein that spans an entire cellular membrane which recognises lipopolysaccharide (LPS) (Poltorak *et al.*, 1998) through the accessory molecule myeloid differentiation factor 2 (MD2) (Kim *et al.*, 2007). The mechanism of LPS recognition is initiated when the acute-phase protein LPS-binding protein (LBP) binds to LPS. The LPS-LBP complex is then transferred and binds to either soluble (sCD14) or membrane-bound CD14 which finally binds to the extracellular domain of TLR4 MD2 followed by TLR4 oligomerisation and activation of the signalling cascade as shown in figure 1.5. Cascade activation involves recruiting adaptor proteins through the signalling domain in the cytoplasm called Toll-Interleukin-1 receptor (TIR) which is present in all TLRs (O'Neill and Dinarello, 2000). Adaptor proteins include TIR-domain containing proteins, myeloid differentiation primary response 88 (MyD88), TIR-associated protein (TIRAP) and MyD88 adaptor-like protein (Mal). The involvement of these adaptor proteins is essential for the activation cascade to take place. The MyD88 activates interleukin-1 receptor-associated kinase 4 (IRAK4) which then activates interleukin-1 receptor-associated kinase 1 (IRAK1) by phosphorylation. IRAK1 and IRAK4 disassociate from the MyD88 and associate with TNF Receptor-Associated Factor 6 (TRAF6). Other molecules including B-cell lymphoma/leukaemia 10 (Bcl10), Mucosa Associated Lymphoid Tissue 1 (MALT1) and Interleukin-1 Receptor-Associated Kinase 2 (IRAK2) also bind to TRAF6 leading to auto ubiquitination (Keating *et al.*, 2007, Sun *et al.*, 2004). After ubiquitination, TAK1-binding protein 1 (TAB2), TAK1-binding protein 3 (TAB3) and TGF-beta activated kinase 1 (TAK1) form a complex with

TRAF6 leading to TAK1 activation. TAK1 molecule then pairs to the I κ B kinase alpha (IKK α), I κ B kinase beta (IKK β) and NF-kappa-B essential modulator protein (NEMO) complex resulting in I κ B phosphorylation followed by the disassociation of I κ B from the P50 and P65 complex. The later complex activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and finally initiates the production of proinflammatory cytokines.

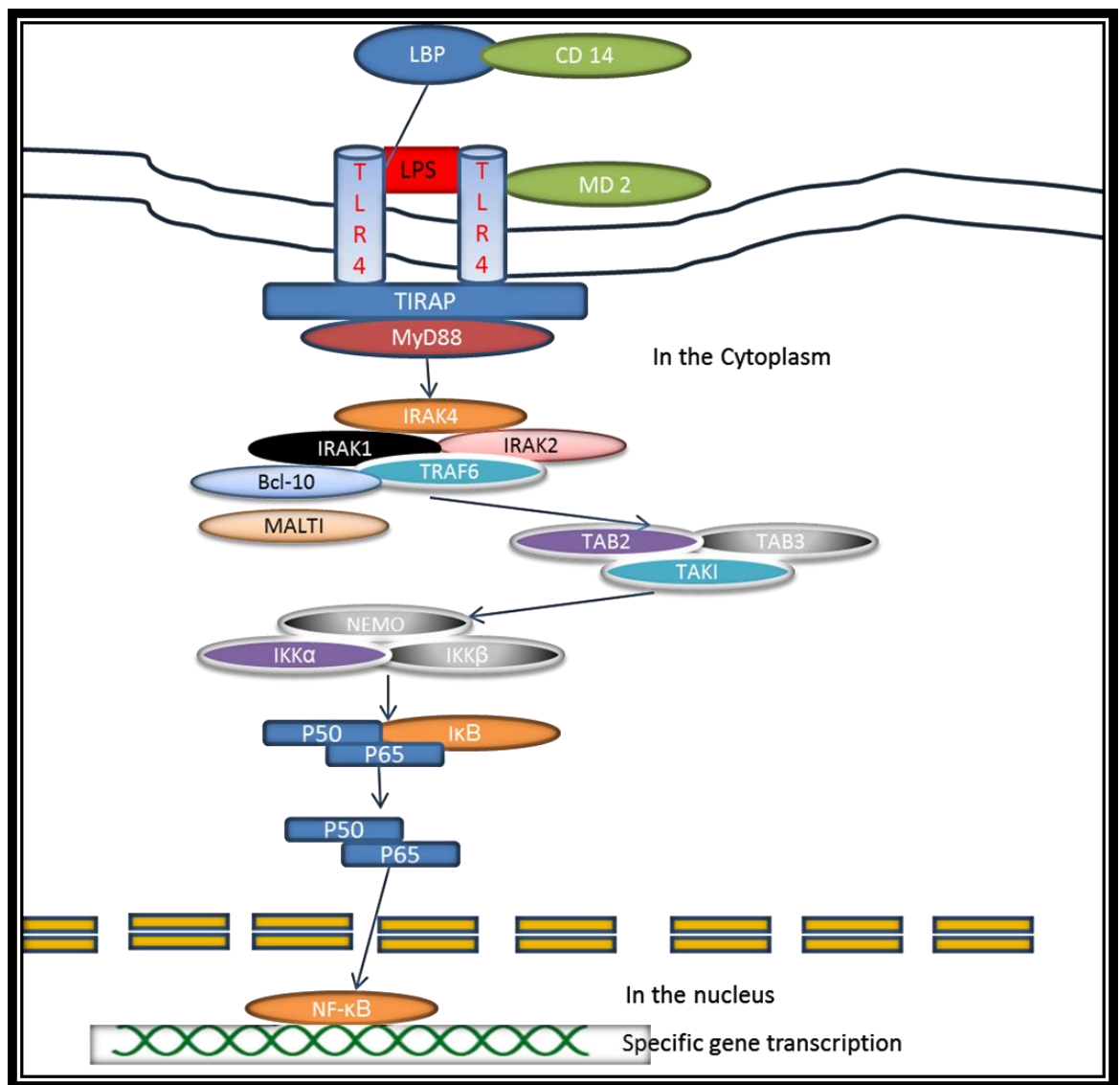


Figure 1.5: A Toll-Like Receptor 4 (TLR4) signalling pathway. TLR4 activates the MyD88-dependent pathway which is responsible for early-phase NF- κ B and MAPK activation that control the synthesis of proinflammatory cytokines. Adapted and modified from Takeda and Akira (2004).

1.6.3 Proinflammatory cytokines

Cytokines are regulators of host responses to both normal and pathological conditions such as infection, immune responses, inflammation, and trauma. Some cytokines act as (proinflammatory), whereas others lessen inflammation and promote healing (anti-inflammatory). In the human body, cytokines are intended to induce biological effects in their local microenvironment where they are produced after exposure to pathological conditions. However, in normal tissues, the occurrence of cytokine-mediated tissue damages is unlikely; mainly because of highly regulated cytokine secretion. In addition, excessive cytokine release from activated cells by LPS for example results in a local tissue injury (Dinarello and Wolff, 1993).

1.6.4 Interleukin-6

Interleukin six (IL-6) is a pleiotropic α -helical cytokine that plays important roles in acute phase reactions, haematopoiesis, bone metabolism (Lakatos *et al.*, 1997), and cancer progression (Berger, 2004). IL-6 secreted by multiple cell types and is encoded on chromosome number one. Moreover, IL-6 has been shown to be endogenous fever inducer in patients with autoimmune diseases or infections. IL-6 is synthesised at sites of inflammation, where it is secreted into the serum and provokes a transcriptional inflammatory response through IL-6 receptor. IL-6 is associated with various inflammation-related disease states, including susceptibility to diabetes mellitus (Kristiansen and Mandrup-Poulsen, 2005) and systemic juvenile rheumatoid arthritis (Nishimoto, 2006).

1.6.5 Interleukin-8

Interleukin-8 (IL-8) was discovered in 1987 as a novel type of neutrophil-activating proinflammatory cytokine that acts as a chemotactant to neutrophils at the inflammation site when released by monocytes and macrophages during exposure to inflammatory stimulants (Baggiolini *et al.*, 1989, Baggiolini and Clark-Lewis, 1992). In addition, it has been suggested that the polymorphism of these genes is associated with rheumatoid arthritis (Troughton *et al.*, 1996) and Alzheimer's disease (Vendramini *et al.*, 2007).

1.6.6 Tumour Necrosis Factor Alpha

The gene coding for TNF- α is located on chromosome 6. TNF- α is a proinflammatory cytokine secreted by a number of cells including activated monocytes and macrophages. TNF- α is considered one of the TNF family of ligands that signals through two receptors, TNFR1 and TNFR2. TNF- α is cytotoxic to various tumour cells and considered an important factor in mediating the immune response against bacterial infections (Curtis *et al.*, 2007). Although TNF- α plays an important role in the activation of innate response, excessive unregulated production of TNF- α might produce pathological changes resulting from chronic inflammation and tissue damage. Elevated levels of TNF- α also play a role in the induction of inflammation and septic shock, as well in the pathogenesis of many chronic diseases such as auto immune diseases, rheumatoid arthritis (Kodama *et al.*, 2005), and diabetes (Shiau *et al.*, 2003).

1.6.7 Interlukin-1 Alpha

The coding gene for IL- α is located on chromosome 2. IL-1 α is one of the interleukin 1 family that is synthesised by stimulated macrophages, neutrophils, epithelial cells, endothelial cells and monocytes. IL-1 α is pyrogenic during an inflammatory response (Dube *et al.*, 2001) and it has multi inflammatory response regulatory functions.

1.7 Rationale for this project

After reviewing the literature and regulations, research gaps and requirements have been identified regarding the current monitoring method for the marine bathing waters. These gaps mainly emerge from the necessity for a rapid method to test the quality of bathing water by untrained water activity performers. In addition, currently used bathing water quality assessment fails to cover the biological relevance to human health of contaminated bathing water; especially the presence of high levels of LPS.

Hence, a general rapid method to assess the bacteriological quality of bathing water in “real-time” is required. Thus total LPS in marine bathing water was here used as a potential general biomarker for bacterial contamination during the summer season of 2012 in the United Kingdom (Chapter 3). In addition, a more specific faecal “real time” indicator is required to assess the faecal contamination. The potential of *Bacteroides* species lipopolysaccharide was investigated as a more specific faecal biomarker to overcome the background level of Gram-negative LPS and to indicate the presence of a recent faecal contamination (Chapter 4). *Bacteroides* species present in systematically collected bathing water were identified and their LPS was profiled for similarity in banding pattern. In addition, the potency of LPS (total and *Bacteroides*) was also investigated in both LAL assay and cell culture models. Furthermore, a method has been designed to specifically detect the LPS of *Bacteroides* in bathing seawater. Bearing in mind the use to date of epidemiological surveys to assess bathing risks; the effect of contaminated bathing water on human health

was investigated by assessing the inflammatory effects of such waters in cell culture models (Chapter 5).

Hence the aim of this research can be divided into several objectives:

- A. To establish a method to measure total LPS in marine bathing water.
- B. To measure the background level of total LPS in bathing water (at a suitable beach).
- C. To isolate, enumerate and correlate number of bacterial indicator CFU from bathing seawater to the total LPS in these waters using the quantitative Kinetic QCL™ LAL assay.
- D. To design an endpoint chromogenic rapid assay for qualitative pass/fail testing the quality of marine bathing water by measuring the levels of total LPS.
- E. To isolate, enumerate, speciate, source track and profile marine sourced environmental *Bacteroides* species, extract LPS present in marine bathing water using classical and molecular methods for future development of LPS-specific indicator method.
- F. To investigate the potential biological relevance of *Bacteroides* LPS in *in vitro* lung macrophage and blood monocyte cell culture models.
- G. To investigate the potential biological relevance of LPS in contaminated bathing water in *in vitro* lung macrophage and blood monocyte cell culture models.

Chapter 2: General materials and methods

2.1 General Reagents preparation

Laboratory reagents and growth media preparations are shown in this chapter. Unless stated otherwise, all laboratory chemicals were acquired from Sigma-Aldrich (Poole, UK), Cell culture reagents and plastic wares were from Lonza (Slough, UK), Sterilin limited (Newport, UK) Greiner Bio-One Ltd (UK), VWR (UK) and Fisher Scientific (UK). Reagents and equipment requiring sterilisation were sterilised by autoclaving (121 °C, 15 min, pressure 15 lbs/in²), while, heat sensitive reagents were sterilised using filtration through either 0.2 or 0.45 µm filters (Sartorius Ltd, UK).

2.2 Microbiological work

All microbiological work was conducted in a class II laboratory, health and safety regulations were followed while conducting all experiment. Generally, all microorganisms were cultured according to the optimal condition requirement; specific conditions for each microorganism are specified when appropriate throughout the chapters in this thesis.

2.2.1 Bacterial growth media

Different bacteria require different medium and optimal conditions for growth, below listed growth media were used to grow microorganisms used in this study.

2.2.1.1 *Bacteroides* Bile Esculin Agar (BBE)

Bacteroides Bile Esculin (BBE) is a selective medium for isolation and presumptive identification of *Bacteroides fragilis* group. BBE contain hemin which is an important growth stimulating factor for most *Bacteroides* species. BBE also contain Esculin, when Esculin is hydrolysed by *Bacteroides* it tends to produce a black zone in the medium around *Bacteroides* colonies which is one of the main presumptive tests for *Bacteroides fragilis* family (Livingston *et al.*, 1978). The ingredients of BBE agar (Atlas, 1997) are shown in the table 2.1.

Table 2.1: Ingredients of *Bacteroides* Bile Esculin (BBE) agar

Ingredients	g L ⁻¹
Oxgall (dried ox bile)	20
TSA*	40
Esculin	1
Ammonium ferric citrate	0.5
Q.S to 1 Litre of distilled water	

TSA* enriched medium containing 1.5% trypticase peptone, 0.5% phytone peptones, 0.5% NaCl, 1.5% agar. TSA supports the growth of many of fastidious microorganisms.

All ingredients except for hemin and gentamycin were dissolved by stirring and heat in distilled water, the pH of the solution was adjusted to 7 then autoclaved at 121 °C for 15 minutes. Hemin stock and gentamicin was prepared separately as explained bellow, 2.5 mL of stock hemin and gentamycin was aseptically added just before pouring the agar in appropriate sterile plates.

2.2.1.1.1 Preparation of hemin solution stock

Hemin stock was prepared by weighting 0.5 g of hemin (Sigma Aldrich, UK) and completely dissolved in 10 mL of 1N NaOH. Volume was Q.S to 100 mL with distilled water then autoclaved at 15 psi pressure 121 °C for 15 minutes.

2.2.1.1.2 Preparation of gentamicin stock solution:

Gentamycin stock solution was prepared by weighting 0.4 g of gentamycin sulphate (Sigma Aldrich, UK) and dissolved in 10 mL of sterile distilled water. Solution was mixed thoroughly then filter sterilised using 0.2 µm syringe filter (Millipore, Ireland).

2.2.1.2 *Bacteroides* Phage Recovery Medium (BPRM)

Bacteroides Phage Recovery Medium (BPRM) was developed by Tartera and Jofre (1987) for recovering *Bacteroides* phages. This broth was used in this study for growing single pure culture *Bacteroides* after colonies development on BBE agar to be used later for LPS extraction and LPS detection assay and further experimental work. The ingredients for the BPRM are shown in table 2.2.

Table 2.2: *Bacteroides* phage recovery medium Ingredients

Ingredient	Weight
Bacteriological peptone	20 g
Tryptone	20 g
Yeast extract	4 g
NaCl	10 g
Monohydrate 1-cystine	1 g
CaCl ₂ anhydrous	0.1 g
MgSO ₄ · 7H ₂ O	0.24 g
Distilled water	955 mL

2.2.1.2.1 Preparation of BPRM broth:

Ingredients were dissolved by stirring using a magnetic stirrer and heat in 955 mL of distilled water and the pH was adjusted to 7, the medium was then distributed into 100 mL screw cap glass bottles then autoclaved at 121 °C for 15 minutes. Additives were added after sterilisation just before using as shown in table 2.3.

Table 2.3: *Bacteroides* phage recovery medium additives

Additive /95 mL	Volume	Sterilisation type
1M Glucose	1 mL	Filter sterilised
1M Na ₂ CO ₃	2.5 mL	Filter sterilised
Hemin stock solution	1 mL	Autoclaved

2.2.1.3 Sugar utilisation medium for *Bacteroides* speciation.

Bacteroides species was initially speciated for their phenotypic characteristics based on Bergey's manual of systematic bacteriology using indole and sugar utilisation test. Certain *Bacteroides* species have the ability to utilise one or more of Rhamnose, Trehalose and Salicin sugars as a carbon source. In order to identify and speciate *Bacteroides* isolates using sugar utilization method, three sugar types were used: Rhamnose, Trehalose and Salicin (Sigma Aldrich, UK). 1 mL of 1 M of each sugar was added per 95 mL of the BPRM with bromophenol blue (10x 1 gram of bromophenol blue dissolved in 10 mL ddH₂O) as a pH indicator for bacterial growth and acid production produced from sugar utilisation.

Each *Bacteroides* isolate was inoculated into 3 different broths supplied with one of the mentioned above sugars. Cultures were incubated in a 48 well plate, in CO₂ at 37 °C. A colour change in the medium from violace to yellowish colour is regarded as a positive result while no colour change is regarded as a negative result. Results then compared to differential characteristics of the *Bacteroides fragilis* species table that was published in Bergey's manual of determinative bacteriology (Bergey and Holt, 1994) and grouped for further molecular identification.

2.2.1.4 Membrane Lauryl Sulphate Broth (MLSB)

Membrane Lauryl Sulphate broth (MLSB) is a medium used for the enumeration of coliforms and *E. coli* in water samples using membrane filtration method. This

medium was purchased and prepared according to manufacturer's instructions (Oxoid, UK). Coliforms colonies develop after 48 hours at 37 °C while *E. coli* develop by incubating the plates for 4 hours at 35 °C then 44 hours at 44 °C. Developed colonies appear as yellow colour colonies after appropriate incubation period.

2.2.1.5 Slanetz and Bartley agar (S and B)

Slanetz and Bartley agar was used for the detection and enumeration of Gram-positive enterococci by the membrane filtration method as recommended by 2006 European Union marine bathing water directive. Slanetz and Bartley agar was purchased and prepared as recommended by the manufacturer's instructions (Oxoid, UK). Enterococci colony developed after incubation for 4 hours at 35 °C to encourage injured or stressed cells then incubate for 44 hours at 44-45 °C. Red-maroon colour colonies were counted as enterococci.

2.2.1.6 Nutrient agar and broth

Nutrient agar is a general purpose medium was used for the cultivation of *E. coli* after initial growing on MLSB in order to obtain pure culture and then inoculums were inoculated into nutrient broth for later LPS extraction and activity determination. Nutrient agar and broth were purchased from Oxoid (UK) and prepared according to the manufacturer's instructions.

2.2.2 *Bacteroides fragilis* NCTC 9343 type culture

Bacteroides fragilis NCTC 9343 type culture was purchased from The National Collection of Type Cultures (NCTC) as a reference for comparison in LPS profiling the LPS from *Bacteroides* species. The *Bacteroides fragilis* NCTC 9343 was revived according to the supplier's instructions.

2.2.3 Calibration curves of *Bacteroides* species and *E. coli*.

A calibration curve is a common method for estimating the number of viable bacterial cells in broth culture by comparing the unknown to a set of standard samples of known concentration. Calibration curves were performed for *Bacteroides* species and *E. coli* isolated from seawater. Randomly selected Inoculums were taken from each agar plate of the *Bacteroides* and *E. coli* and inoculated in a suitable broth medium (BPRM for *Bacteroides* growth and nutrient broth for *E. coli* growth) then incubated in optimum growth conditions. Optical density of a linear dilution was determined using a spectrophotometer (Helios Epsilon spectrophotometer) at 590 nm. After that, a tenfold serial dilutions (showed in figure 2.1) were prepared the next day from broth cultures, 100 µL were plated on suitable agar in duplicate and incubated aerobically at 37 °C for *E. coli* and 37 °C anaerobically for *Bacteroides*. After colony development, a simple formula was applied to obtain the number of viable bacterial cells in the undiluted broth culture as follows:

$$\text{Original bacterial number} = \frac{\text{number of CFU}}{\text{volume plated}} \times \frac{1}{\text{dilution factor}}$$

A standard curve was then constructed by plotting the bacterial viable count to the optical density. This standard curve can be used to determine the viable number of bacteria in unknown broth culture by measuring the optical density of that sample and refer to the calibration curve.

2.2.4 Cryopreservation of bacterial isolates in liquid nitrogen culture collection

In order to preserve *Bacteroides* isolates from seawater for further use in future and to maintain a stock culture using cryopreservation. *Bacteroides* isolates including *Bacteroides fragilis* type culture NCTC 9343 were subcultured in 30 mL of BPRM broth and incubated in CO₂ incubator for 48 hours. Bacterial cells were harvested by centrifuging the culture for eight minutes at 4000 RPM. After centrifuging, supernatant was discarded in freshly prepared 2% Virkon solution and left for 20 minutes then discarded properly. Pellets were re-suspended with one mL of 30% sterile glycerol, BPRM was used as diluent. The suspension was divided into two 1 mL cryovials (Fisher Scientific, UK). Cryovials were immersed in liquid nitrogen for snap freezing then placed in liquid nitrogen tank after taking necessary safety measures.

2.3 Lipopolysaccharide analysis methods

Lipopolysaccharide analysis is a complex task in terms of physical structures and activity. Although the main structure of LPS in different Gram-negative bacteria usually have similar LPS “backbone”, however, it seems that the different Gram-negative bacteria possess different structural and variable activity levels. In this study, it has been focused on studying the LPS of *Bacteroides* species as a potential faecal biomarker in marine bathing waters (Chapter 4). LPS analysis includes:

1- Analysis of *Bacteroides* LPS structure: analysis of structure was conducted to explore LPS banding pattern in the SDS-PAGE method and compared to *E. coli* reference LPS.

2- Analysis of *Bacteroides* LPS activity: the activity of *Bacteroides* LPS was investigated using two methods:

A- The activity of *Bacteroides* LPS using the Limulus Amebocyte Assay (LAL, chapter 3 and 4).

B- The activity of *Bacteroides* LPS stimulation of human monocytic Mono Mac 6 (MM6) and mouse macrophage MPI cells (Chapter 4).

2.4 Mammalian cell culture models

2.4.1 Max Planck Institute (MPI) cells

Max Planck Institute (MPI) cells are newly described self-renewing, GM-CSF dependent, non- transformable, lung alveoli macrophage like which grow as adherent and suspension cells (Fejer *et al.*, 2013). MPI cells were grown in Roswell Park Memorial Institute medium (RPMI) 1640 with L-Glutamine, 10% FCS, 1% Pen-Strep (1000 U penicillin per mL and 1000 U streptomycin per mL), 1% L-Glutamine (200 mM in 0.85% NaCl solution) and 10 μ L of GM-CSF (30 ng mL⁻¹). MPI cells were split every 2-3 days once reach 80% confluency. MPI cells were washed twice with Ca²⁺ and Mg²⁺ free PBS then cells detached using cell culture grade ethylenediaminetetraacetic acid (EDTA, Lonza, UK), a chelating agent used to lift some cell types from the culture vessel surface into suspension. After 5 minutes incubation at 37 °C under 5% CO₂ / 95% air in humid atmosphere incubator, cells were aspirated using cell culture grade pipette into Falcon™ tube and spun at 160 x g for 5 minutes. Supernatant was discarded in 1% freshly prepared Virkon, followed by suspending the cells with appropriate volume of medium and cells were counted using standard cell counting method.

2.4.2 Mono Mac 6 (MM6) cell line

Mono Mac 6 (MM6) are monocytes originally isolated from the peripheral blood of a 64 year old man with relapsed Acute Monocytic Leukaemia (AML) in 1985 (Ziegler-Heitbroc *et al.*, 1988). MM6 cell line was obtained from the German

collection of human and animal cell cultures (Braunschweig, Germany). MM6 cells are suspension cell line, they secrete large amounts of IL-6 and also TNF- α into the conditioned medium when stimulated with LPS and chosen because of their ease of processing and low variability. MM6 were grown in surface treated filter cap 75 cm² flasks (Greiner, UK) in 90% RPMI-1640 (BioWhittaker®, Lonza, UK) supplemented with 10% (v/v) foetal calf serum, 2 mM L-glutamine, 1% non-essential amino acids, 1 mM Sodium pyruvate all supplied by Lonza (Slough, UK) and incubated at 37 °C under 5% CO₂ / 95% air in humid atmosphere incubator. MM6 cells were examined by inverted microscope (Olympus CK40, UK), passaged every seven days and subcultured every 2 days. MM6 cells were subcultured by gently aspirating the culture medium using 10 mL individually wrapped pyrogen-free plastic pipette (Sterling and Greiner Bio-One Ltd, UK) into 50 mL pyrogen-free polypropylene Falcon™ tubes (VWR, UK) and centrifuge at 160 x g for 5 minutes. Supernatant was carefully aspirated without disturbing cell pellet. Fresh RPMI medium was added and cells were counted manually using a haemocytometer by carefully loading a few microliters of cell suspension and left to settle for 2 minutes before counting. Four Squares were counted and the mean was obtained then multiplied by 10⁴. Cell density was kept between 0.3 - 1 × 10⁶ cells mL⁻¹. Assessing cells viability before running an experiment was conducted using trypan blue dye exclusion method and cell viability of each experiment was more than 97%. 0.4% trypan blue was prepared by weighing 0.4 g of trypan blue then added to 80 mL of PBS, heated to complete dissolve then left to cool down, the volume of solution was then topped up to 100 mL PBS.

2.4.3 Mammalian cells counting and cells viability using a haemocytometer

A clean haemocytometer and cover slip assembled by mildly moistening the haemocytometer and affixing the cover slip, paying attention to the presence of Newton's rings. 100 μL of cell suspension was gently mixed with 0.4% trypan blue reagent then using a pipette appropriate volume was gently loaded into the haemocytometer. Haemocytometer grid was visualised under the microscope and a total of 4 large corner squares were counted paying attention to record the number of dead and alive cells. The difference between live and dead cells is that live cells appear colourless while dead cells stain with the trypan blue dye. To calculate the cell count per mL:

$$\text{Total cell count mL}^{-1} = \text{the average number of cells} \times 10^4$$

Cellular viability was determined using the following formula:

$$\text{Viable cells (\%)} = \frac{\text{total number of viable cells per ml of aliquot}}{\text{total number of cells per ml of aliquot}} \times 100$$

2.4.4 Cryopreservation of mammalian cells

MPI Cells were grown to 80% confluence in order to prepare stocks for long term storage. Cells were cultured in 75 cm^2 tissue culture flasks, detached with EDTA and harvested by centrifugation at $160 \times g$ for 5 minutes, the supernatant was aspirated and the pellet was gently resuspended, counted and adjusted to give a final concentration of 4×10^6 cells mL^{-1} in storage medium. Storage medium contains 20% FBS and 10% dimethyl sulfoxide (DMSO). Aliquots of 1

mL suspension were placed in 2 mL cryovials and incubated at -80 °C overnight in insulated boxes. Afterwards, vials were transferred into liquid nitrogen tanks for long term storage.

In order to cryopreserve the MM6 cells, cell counts and viability were assessed using the above mentioned trypan blue exclusion method then resuspended after spinning at 160 x g for 5 minutes in 80% RPMI and 20% FCS at concentration 4×10^6 cells per mL. An equal volume of 60% RPMI 20% FCS, 20% DMSO was gradually added drop by drop while swirling the tube to assure proper mixing and avoiding cellular stress. one mL aliquots were transferred into cryovials, placed in a Styrofoam box at -80° C freezer overnight then transfer to liquid nitrogen for long term storage (Ziegler-Heitbroc *et al.*, 1988).

2.5 Principle of Enzyme Linked Immunosorbent Assay (ELISA)

The Enzyme Linked Immunosorbent Assay (ELISA) is a powerful method for detecting and quantifying a specific molecule usually in a complex mixture. ELISA was developed by Engvall and Perlmann in 1971. The principle of this method is to immobilise the molecule of interest either by direct adsorption to a plate with the appropriate buffers or using capture antibodies specific to the molecule of interest adsorbed to surface-treated microplates such as MaxiSorp® (Nunc, Thermo Scientific, UK). The molecule of interest is then detected using one or more detection antibodies depending on the ELISA format. After the formation of the immune complex, an enzyme, usually horseradish peroxidase

(HRP), involved chromogenic reaction occurs when an appropriate substrate is added to the reaction well. This chromogenic reaction is proportional to the concentration of the molecule of interest. A washing step with washing buffer is performed to ensure any non-specific binding or excess of unbound reagents is removed.

The principle of the chromogenic reaction is that the substrate oxidised by the HRP conjugated secondary antibodies in the ELISA wells produces a blue coloured solution, changed to a yellow coloured solution when the reaction is stopped with 1 N H_2SO_4 , maximum absorbance is read at 450 nm. Similarly, super aqua blue (3-ethylbenzthiazoline-6-sulfonic acid) is another substrate used in this study that undergoes oxidizing reaction by the HRP resulting in a soluble blue-green colour end product, whose absorbance is measurable using spectrophotometer at 405 nm. The main reason in using ELISA is because of high sensitivity, ease to perform and small sample volume required for the assay. ELISA has a variety of format, the most used formats are summarised in figure 2.1.

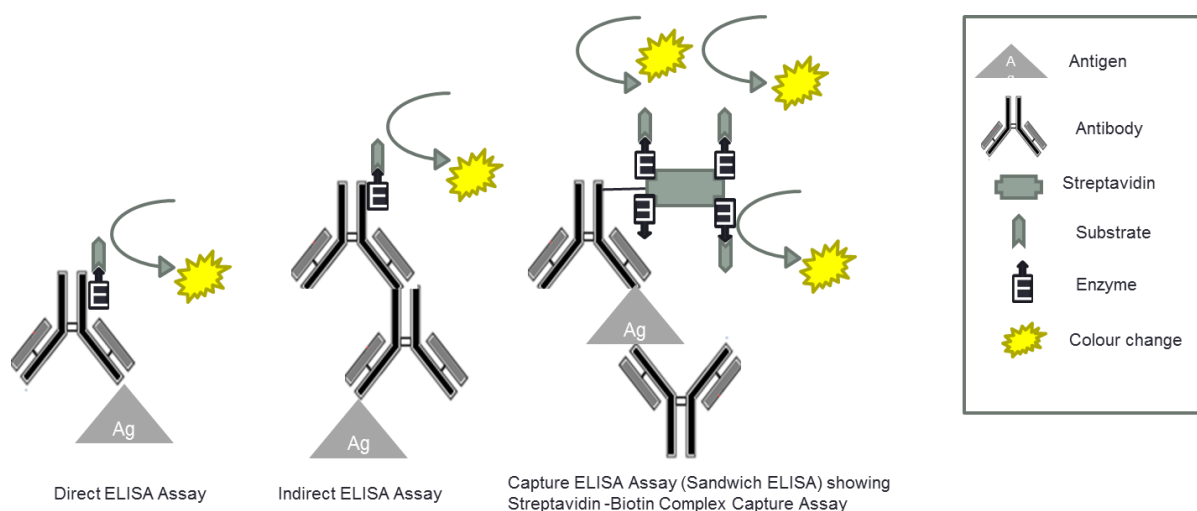


Figure 2.1: Common Enzyme Linked Immunosorbent Assay (ELISA) formats.

Each ELISA format has a particular advantage; however, the sandwich ELISA has a high sensitivity, specificity and low cross reactivity compared to the other ELISA formats. In addition, the streptavidin-HRP bridging in sandwich ELISA amplifies the signal as it can accommodate four HRP molecules instead of one in most other formats.

2.5.1 Checkerboard ELISA

All sandwich ELISA assays conducted in this study were optimised using checkerboard titration method in order to obtain the highest signal/noise ratio with the optimum concentration of capture and detection antibodies.

Checkerboard ELISA is a method that involves the dilution of two reagents against each other in order to optimise the best combination that gives the highest signal to noise ratio. The maximum number of reagents that can be titrated on a plate is two. The checkerboard method was used to optimise the

concentrations antigen, capture and detection antibodies. The checkerboard method was followed as described in the ELISA guidebook (Crowther, 2000).

2.6 Statistical analysis

Numeric values were expressed as mean \pm standard error of the mean unless stated otherwise. All numerical data were analysed using GraphPad Prism version 5, Primer 6 version 6.1.10 and WinKQCL endotoxin detection and analysis software version 3.0.1 (Lonza).

**Chapter 3: The Potential of LPS as a real-time
biomarker of faecal contamination in marine bathing
waters**

3.1 Introduction

The quality of beaches in the UK and Europe is governed by the European Union Bathing Water Directive 2006/7/EC (2006) which sets legislation and defines standards that all beaches must comply with in order to be considered as designated bathing beaches. Faecal bacteria, including *Escherichia coli* (*E. coli*) and enterococci, are important indicators for determining the quality of marine bathing waters as they can eliminate the need for expensive and time consuming testing for specific pathogenic organisms (Lucena *et al.*, 1994). The previously used faecal indicator bacteria (FIB) were total coliforms, faecal coliforms, *E. coli* and faecal streptococci as these microorganisms can provide a general indication of faecal pollution. However, these were later revised and coliforms were substituted by enterococci. European Bathing Water Directive (2006) has divided the quality into four ranks (excellent, good, sufficient and poor) depending on the number of FIB present in a 100 mL of bathing water sample (Table 1.2).

However, current quantitative culture-based FIB methods have drawbacks (Anderson *et al.*, 2005, Borrego *et al.*, 1983, Field and Samadpour, 2007) as these indicators have a tendency to multiply in bathing seawater and the results are retrospective, taking at least 24-48 hours to inform regulatory bodies. Marine recreational water users require an instant, qualitative indication of possible bacterial pollution events in order to make an informed decision on whether to undertake an activity. LPS has been targeted by previous studies for estimating total biomass and testing potable water (Jorgensen *et al.*, 1976,

Watson *et al.*, 1977, Evans *et al.*, 1978, Jorgensen *et al.*, 1979, Haas *et al.*, 1983) but not as a potential biomarker for bacterial-polluted bathing water. To our knowledge, there is no evidence of a rapid quantitative method to evaluate the bacteriological quality of marine bathing water.

The aim of this chapter was to measure the *total* LPS levels in seawater samples and correlate them to the current culture-based methods for determining this water quality using FIB such as *E. coli*, total coliforms and *Bacteroides*. This was undertaken at a surfing/bathing beach during the UK bathing season, including a heavy rainfall pollution event.

3.2 Materials and methods

3.2.1 Topography of the study area and seawater samples collection

Challaborough beach is a popular bathing, body boarding and surfing beach located in the District of South Hams on the south coast of Devon, UK (Latitude: 50.287159, Longitude: -3.899052). It is a horseshoe shaped bay virtually divided by a small stream that runs from a valley down into the sea (Figure 3.1). This beach was particularly chosen because of its occasional pollution events that take place on stormy days.

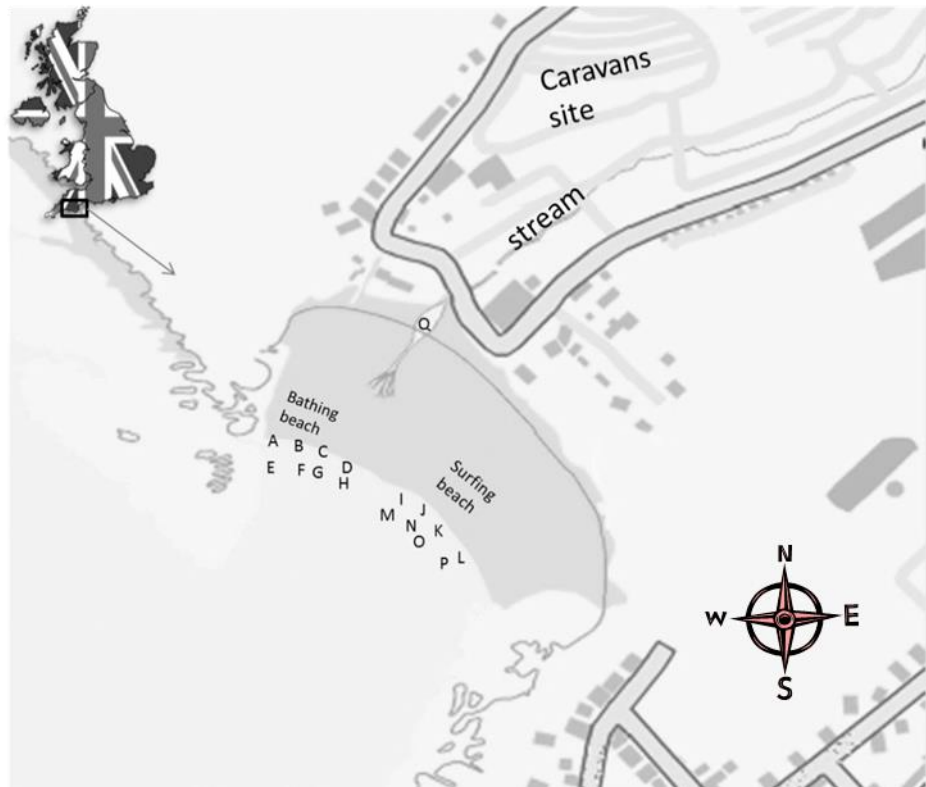


Figure 3.1: Topography of Challaborough beach at low tide and the surrounding caravan site. A, B, C and D represent sampling sites for shallow water and sediment at the bathing area. E, F, G and H represent sampling sites of approximately 1 metre depth water sampling in the bathing area, I, J K and L represent sampling sites for shallow water and sediment at the surfing area. M, N, O and P represent sampling sites of approximately 1 metre depth water sampling in the surfing area. The “Q” represents sampling site for stream water.

Challaborough village comprises two small fixed caravan sites and a few private houses which end just beside the beach. This beach was chosen because it is divided into two distinct areas: bathing and surfing.

Composite water samples for each station were collected on 10 occasions over the summer of 2012 at Challaborough beach starting from early June to the beginning of September (UK bathing season); in addition to water samples from the stream. Water samples were taken usually once a week with a random timing for low and high tide except for one sample in which heavy rainfall and

high level of runoff had occurred and which was considered as the “Highly contaminated” sample. Samples were collected in disposable, sterile, screw capped wide mouth pots for the bacteriological investigation whilst 50 mL pyrogen-free Falcon™ tubes were used to collect water for LPS detection. Shallow water samples were collected from the surface of the water whilst deeper water samples were taken from approximately one metre depth. Sediment samples were also collected in similar pots from the shallow water area for both bathing and surfing areas. Samples were immediately taken to the laboratory and processed within 3 hours. Samples for LPS detection were aliquoted into LPS-free glass tubes (Lonza, UK) and preserved at -20 °C until assayed.

3.2.2 Seawater samples filtration

After swirling by hand for three minutes for homogenisation, water samples were filtered aseptically through a 0.45 µm membrane (Whatman, UK) and placed on appropriate media; *Bacteroides* Bile Esculin (BBE) (Livingston *et al.*, 1978), Membrane Lauryl Sulphate Broth and Slanetz and Bartley agar (Oxoid, UK) to isolate *Bacteroides*, total coliforms/*E. coli* and enterococci respectively. Appropriate volumes of the samples were aseptically filtered in duplicate using a vacuum pump attached to the water filtration system (Fisher Scientific Ltd., Loughborough, UK). Sediment samples were prepared by 1:10 dilution of the sediment in sterile synthetic seawater commercially known as Instant Ocean (Underworld, UK) and placed in stomacher bags, then placed in a stomacher (Seward Lab, UK) for 2 minutes, and left to settle for 10 minutes. Samples were then filtered as described above. BBE agar cultures were incubated in an

anaerobic incubator (Don Whitley, UK) at 37 °C for 48-72 hours, Slanetz and Bartley agar was incubated aerobically at 35 °C for 4 hours then at 44 °C for 44 hours and Membrane Lauryl Sulphate total coliforms cultures was incubated aerobically for 24-48 hours and for *E. coli* incubated aerobically at 35 °C for 4 hours, then at 44 °C for 44 hours.

3.3 Bacterial culture maintenance and bacterial enumeration

The number of colony forming units (CFU) was enumerated and numbers were expressed as CFU 100 mL⁻¹ of water and CFU g⁻¹ of sediment after appropriate incubation periods. Bacterial cultures were subcultured on a regular basis to ensure their viability in order to be used in different experimental work.

3.4 Kinetic QCL™ LAL assay procedure

Testing the activity of total lipopolysaccharide in marine bathing waters was carried out using a highly sensitive Kinetic QCL™ LAL assay. Kinetic QCL™ LAL assay was purchased from Lonza, UK (catalogue number 50-650U) and was optimised to suit the purpose of this study.

The principle of LAL assay is discussed in section 1.5.2. Briefly, a sample containing LPS from Gram-negative bacteria is mixed with LAL substrate (Ac-Ile-Glu-Ala-Arg-pNA) in appropriate condition and monitored over a period of time for the appearance of a colour change due to LPS presence. Reaction time is the time that a colour change (yellow) reaches an optical density of 0.2; this

reaction time is inversely proportional to the concentration of LPS and the concentration of unknown samples is determined from standard curve.

The Kinetic QCL™ LAL assay kit supplied with vials containing a lyophilised amoebocyte and chromogenic substrate (LAL reagent). A template was created using the WINKQCL (Lonza) software prior to running the LAL assay then a 100 µL samples of appropriate bathing water dilutions and tenfold range standard LPS (0.005-50 EU mL⁻¹ of *E. coli* O55:B5 LPS) were loaded in duplicate into LPS-free 96 well plates (Costar, Corning, USA) and incubated for 10 minutes at 37 °C in a pre-warmed plate reader (BioWhittaker elx808, BioTek, UK). LAL reagents were reconstituted immediately before use with 2.6 mL LPS-free water provided with the kit, poured into LPS-free reservoir and 100 µL was added per well using a multichannel pipette and then the plate was loaded again into the plate reader to start the experiment. The default template parameters of WinkQCL endotoxin detection and analysis software version 3.0.1 (Lonza) were set to 40 readings with a 405 nm measurement filter. Concentrations of unknown samples were calculated using the values of the standard curve to give a quantitative LPS (EU mL⁻¹) value. All tips (Fisher scientific, UK) and glass dilution tubes (Lonza, USA) used in each experiment were certified as LPS-free.

3.5 Seawater samples dilutions

Composite water samples of the five water areas were prepared by mixing one mL on each individual water 10 samples from each area into a LPS-free glass tube and then mixed well. Ten-fold serial dilutions were prepared for each

composite water sample and assayed for their LPS activity using the Kinetic QCL™ LAL assay.

3.6 LPS Spike and recovery assay

A spike and recovery assay was conducted to assess whether there is any inhibition/enhancement when assaying the activity of LPS present in seawater. Instant Ocean (Underworld, UK) was spiked with a final concentration of 0.5 EU mL⁻¹ *E. coli* O111:B4 LPS (Lonza) and then a KQCL™ LAL assay was run for LPS recovery. Appropriate standards and controls were included whilst running the assay including positive and negative controls.

3.7 Optimisation of an endpoint chromogenic LAL assay

After showing that the total LPS measurement in the marine bathing waters highly correlates to the number of FIB, and after setting the threshold above which the bathing water is suggested as unsuitable for bathing; an endpoint chromogenic LAL assay (Genscript, USA) was optimised to measure total LPS in marine seawater to be used *in situ* without the need for sophisticated equipment. This method is a highly sensitive method with a sensitivity of 0.005–1 EU mL⁻¹. Water samples were diluted 1/100 in LPS-free water then 100 µL of this water sample was added into LPS-free glass tube. 100 µL of the LAL reagent was added to the water sample and incubated for 10 minutes at 37 °C, this was achieved by using commercial multi use hand warmers gel packs that

were held in the hand to provide the heat necessary to complete the LAL assay reaction. A thermometer was incubated similarly to the tube to assure that 37 °C was achieved. After incubation, 100 µL of the LAL substrate was added into the reaction tube and incubated again for 6 minutes at 37 °C. 500 µL of stop solution, colour development 1 and colour development 2 were added in that order which led to a colour change from colourless to purple colour, the deeper the colour the higher levels of LPS present in the water sample. Negative (LPS-free water) and positive (0.5 EU mL⁻¹) controls were run simultaneously with each LAL assay run for quality assurance and comparison. Water samples investigated for their FIB numbers and correlated to the LPS colour intensity.

3.8 Statistical analysis

Statistical analysis was performed using Microsoft office Excel 2010 and Minitab® version 16.1.1 (Kruskal-Wallis test and one-way analysis of variance). LAL assay concentrations were calculated using WinKQCL endotoxin detection and analysis software version 3.0.1 (Lonza). A $p < 0.05$ was considered significant.

3.9 Results

3.9.1 Faecal indicator bacteria enumeration

Colony forming units of *E. coli*, *Bacteroides*, enterococci and total coliforms isolated from collected bathing waters from the five areas over the water sampling collection period were enumerated and expressed as CFU 100 mL⁻¹ in figures 3.2, 3.3, 3.4 and 3.5.

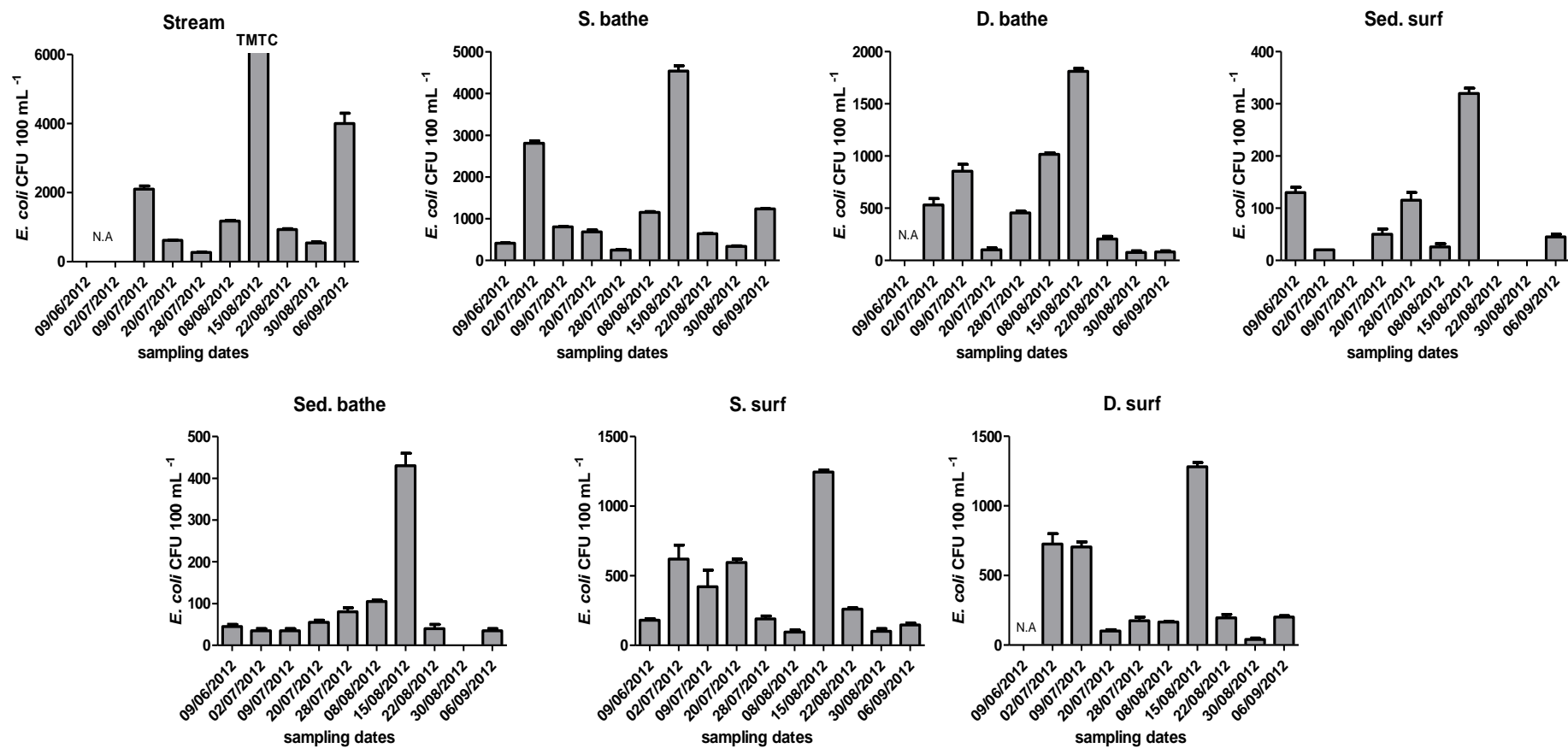


Figure 3.2: Mean number of CFU 100 mL⁻¹ of *E. coli* present in seawater samples collected from the five areas of shallow and deep bathing and surfing (S and D) and the stream in addition to sediment samples (Sed.) from the bathing and surfing areas. N= 4, error bars represent the standard error of the mean. TMTC= too many to count.

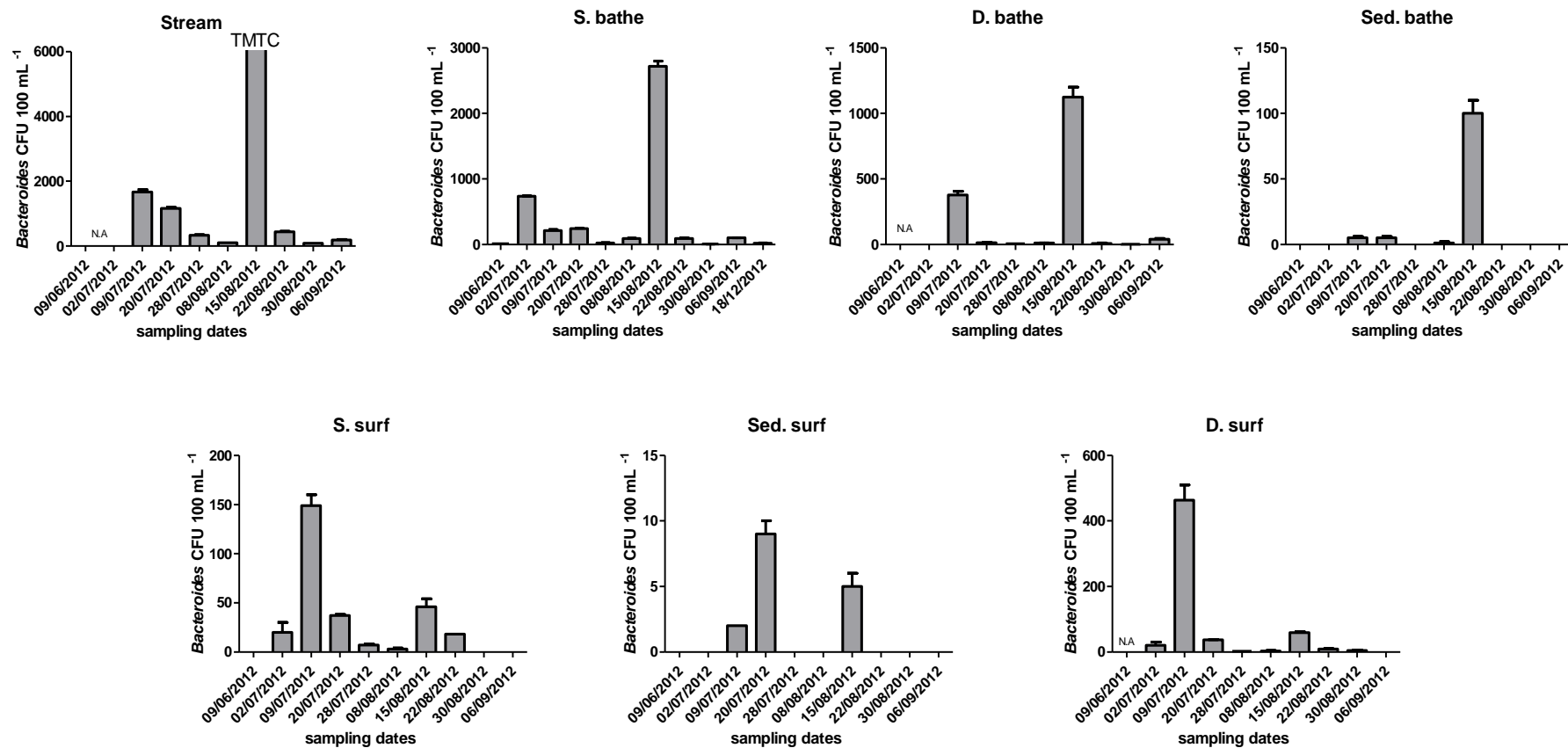


Figure 3.3: Mean number of CFU 100 mL⁻¹ of *Bacteroides* species present in seawater samples collected from the five areas of Shallow and deep bathing and surfing (S. and D.) and the stream in addition to sediment samples (Sed.) from the bathing and surfing areas. N= 4, error bars represent the standard error of the mean. TMTC= too many to count.

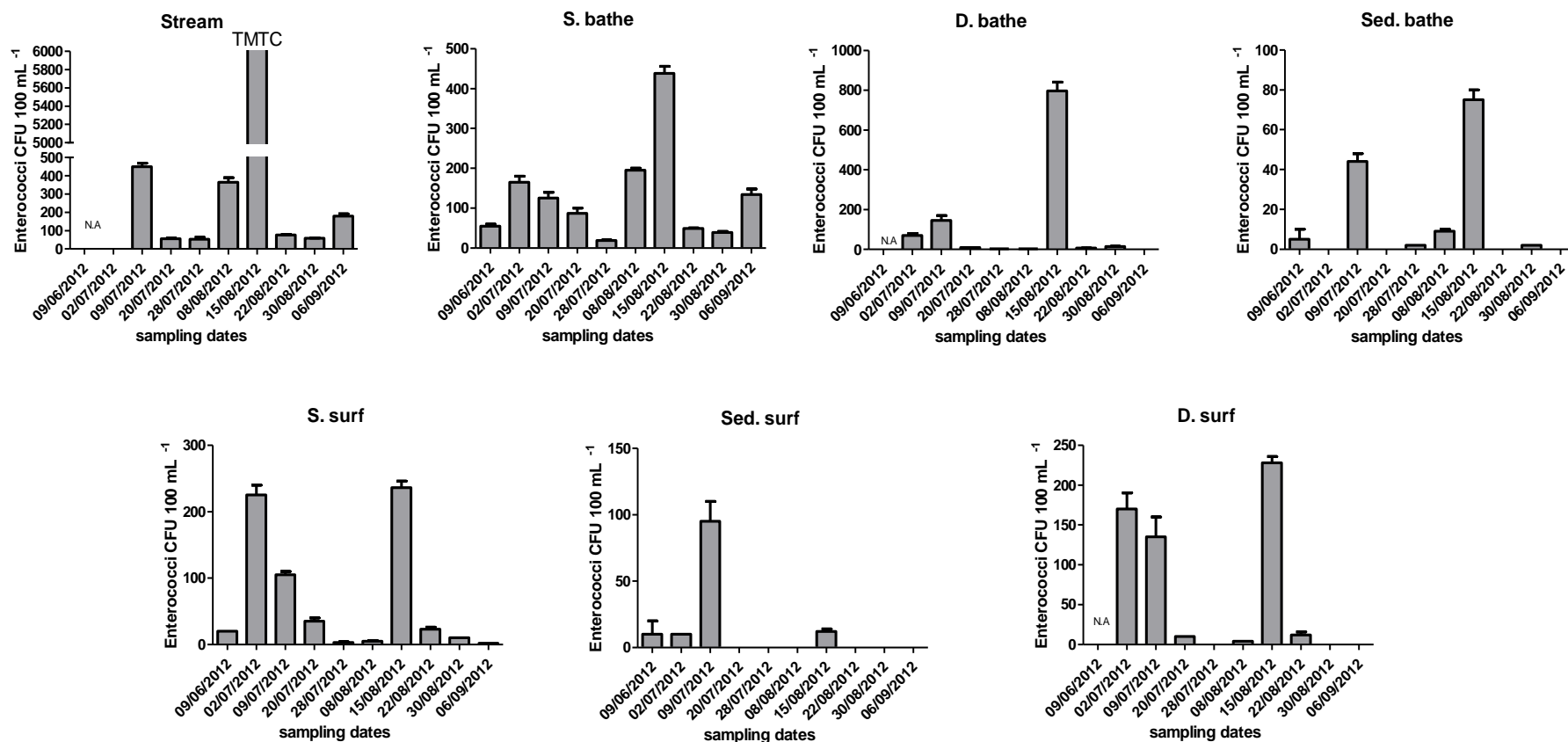


Figure 3.4: Mean number of CFU 100 mL⁻¹ of enterococci present in seawater samples collected from the five areas of Shallow and deep bathing and surfing (S. and D.) and the stream in addition to sediment samples (Sed.) from the bathing and surfing areas. N= 4, error bars represent the standard error of the mean. TMTC= too many to count.

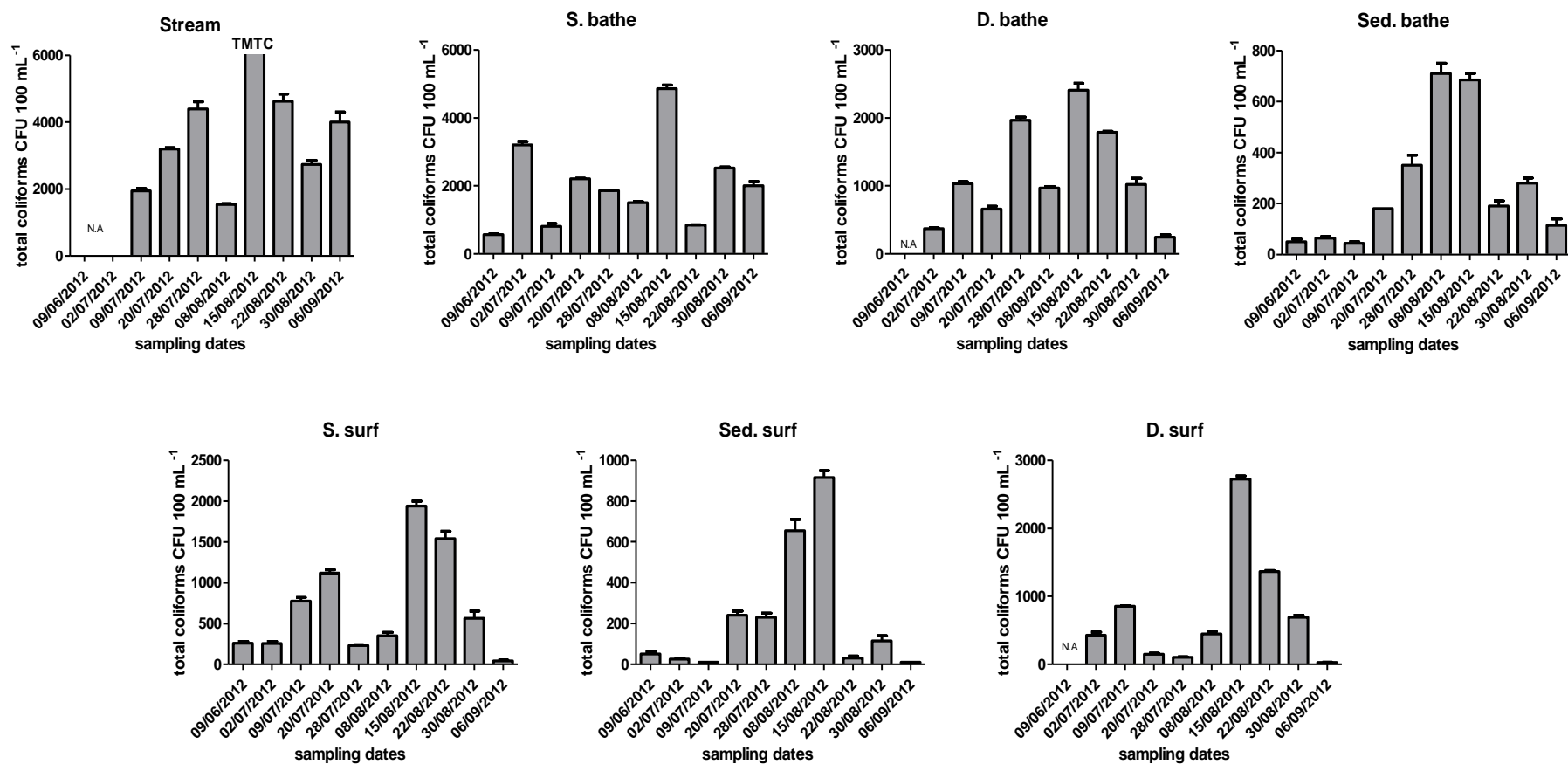


Figure 3.5: Mean number of CFU 100 mL⁻¹ of total coliforms present in seawater samples collected from the five areas of Shallow and deep bathing and surfing (S. and D.) and the stream in addition to sediment samples (Sed.) from the bathing and surfing areas. N= 4, error bars represent the standard error of the mean. TMT= too many to count.

In order to make an easier comparison, the mean CFU in the 10 composite samples of the four areas for *Bacteroides*, *E. coli*, enterococci and total coliforms over the sampling period is shown in figure 3.6. The stream area results are not shown in the composite samples because of the high levels of enumerated bacteria, and also because the stream is not regarded as designated bathing water. [In addition, the levels of FIB were too many to count in some of stream water samples hence it was not included in figure 3.6].

The number of indicator bacteria generally showed that the bathing area was more contaminated than the surfing area. There was a significant difference in the number of CFU of *E. coli* ($p = 0.03$) and total coliforms ($p = 0.01$) when comparing the surfing and the bathing area.

Sediment samples showed extremely low numbers of bacteria; it consists of small rocks and sand grains which tend to have a poor surface for bacterial attachment and eventually a bacterial reservoir (Harrison, 2012) therefore sediment results were ignored. Results showed that there is a pattern in terms of bacterial distribution; the stream runs through the valley and between the tourist caravan site and it pours into the bathing area, so the bathing area is the most affected area on the beach and showed the highest number of *Bacteroides*, *E. coli*, enterococci and coliforms among the rest of beach areas which shows that the stream is the main source of contamination. As soon as the stream enters the bathing area, the water becomes diluted and distributes to the other areas of the beach.

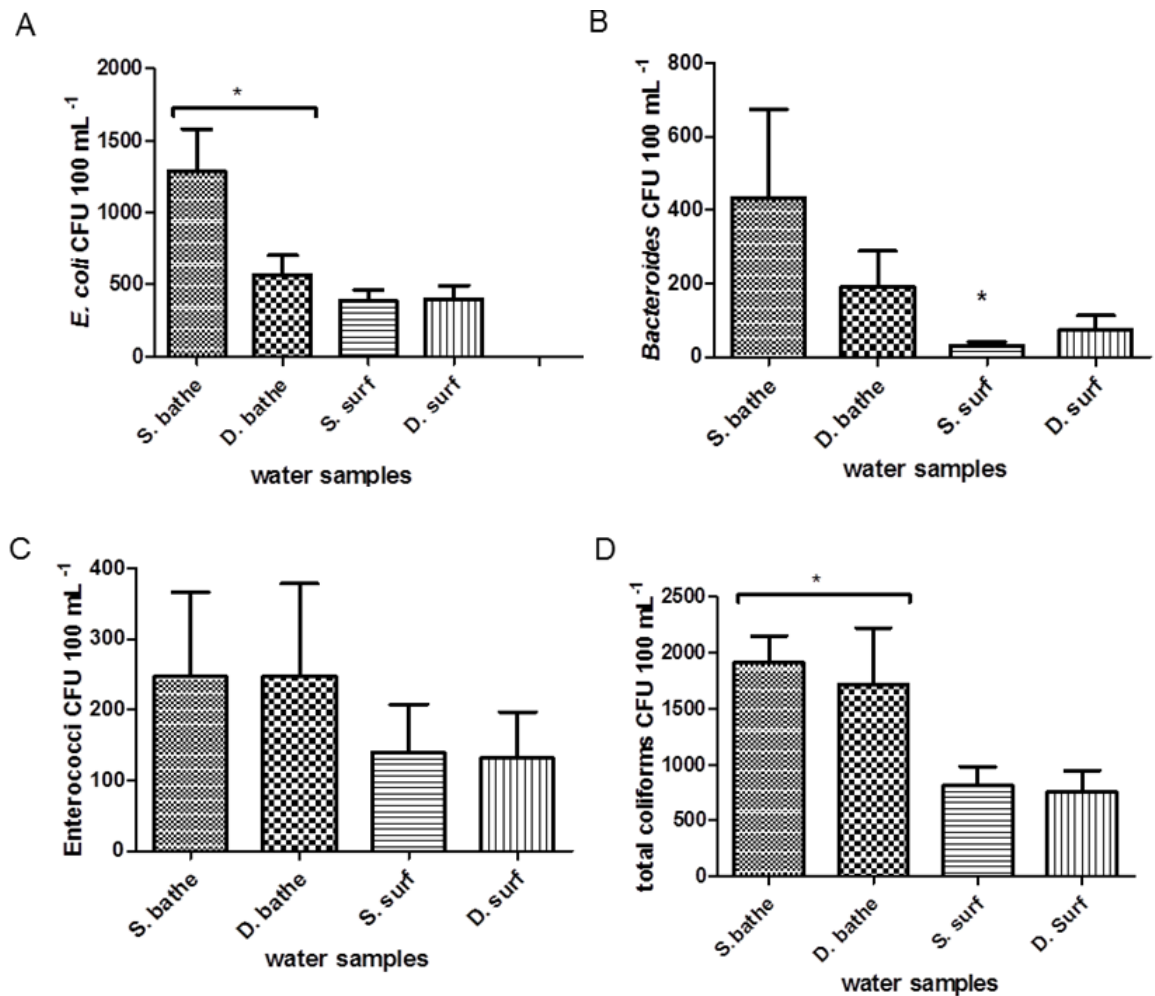


Figure 3.6: Mean number of CFU100 mL⁻¹ bacteria in 10 composite bathing water samples for four areas. (A) *E. coli*, (B) *Bacteroides* species, (C) enterococci and (D) total coliforms. S. bathe represent shallow water samples collected from the bathing area, D. Bathe represents deep water samples collected from the bathing area, S. surf represents shallow water samples collected from the surfing area, D. surf represents deep water samples collected from the surfing area. n=10. Bars show standard error of means. (*) $p < 0.05$ using Kruskal-Wallis test. *E. coli* $p < 0.05$ vs S. surf and D. surf. *Bacteroides* $p < 0.05$ versus S. bathe. Total coliforms $p < 0.05$ vs. S. surf and D. surf.

Bacteroides results only showed a significant difference between S. surf and S. bathe area while enterococci showed no significant difference between the bathing and the surfing area, however, the numbers of CFUs were still higher in the bathing area. Sediment samples showed extremely low numbers and hence results are not shown. A 95 and 90 percentile was calculated according to the

recommended formula by the 2006 European directive as shown in 2 formulas below:

$$\text{Upper 95 – percentile} = \text{antilog} (\mu + 1.65 \sigma)$$

$$\text{Upper 90 – percentile} = \text{antilog} (\mu + 1.282 \sigma)$$

The log₁₀ value of all bacterial concentrations in the data sequence to be evaluated was taken then the arithmetic means (“μ”) of the log₁₀ values taken. Standard deviation of the log₁₀ values (“σ”) was calculated. Afterwards, formulae were applied to derive the upper 90 and 95-percentile point of the data probability density function using formula above. Results showed that all water samples from both bathing and surfing areas were classified as “poor”.

3.9.2 Determination of total LPS activity in marine water samples using Kinetic QCL™ LAL assay

A 5 tenfold serial dilution of LPS standards was prepared and included in every LAL assay in order to construct a standard curve and levels of LPS were reported in endotoxin units/ millimetre (EU mL⁻¹). An example of a LAL assay calibration curve is shown in figure 3.7.

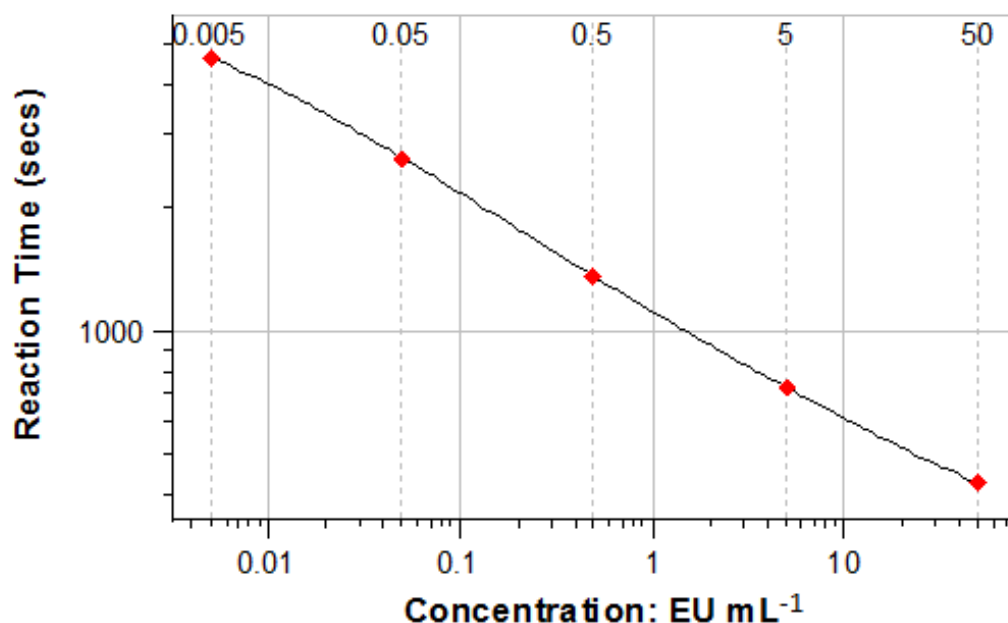


Figure 3.7: a log/log linear correlation of the reaction time of each LPS standard corresponding its LPS concentration obtained using WinKQCL software version 3.0.1. This LPS standard curve ranging from 0.005 to 50 EU mL⁻¹ showing a linearity of -0.999

Composite samples of the ten water samples from each area i.e. shallow or deep (S or D), bathing or surfing (bathe or surf) and the stream were prepared. Four dilutions (undiluted, 1/10, 1/100 and 1/1000) from each composite sample were assayed for LPS activity using Kinetic QCL™ LAL assay (Lonza, UK) according to the manufacturer's instructions and results are shown in table 3.1.

Table 3.1: LAL assay results of composite water sample of the five sampling areas: stream, shallow and deep bathing and surfing areas expressed in EU mL⁻¹. n=4, results represent mean \pm standard deviation of the mean.

Composite water samples						
Dilutions		Stream	S. bathe	D. bathe	S. surf	D. surf
	Undiluted	> 50	8.35 ± 0.15	4.36 ± 0.103	2.89 ± 0.078	2.66 ± 0.048
	1/10	13.8 ± 0.35	5.83 ± 0.019	4.75 ± 0.002	2.75 ± 0.004	2.40 ± 0.005
	1/100	1.35 ± 0.002	0.586 ±0.016	0.485 ± 0.001	0.451 ± 0.001	0.337 ± 0.001
	1/1000	0.135 ± 0.003	0.0688 ± 0.02	0.0356 ±0.122	0.0347 ± 0.037	0.0271 ± 0.002

Interestingly, undiluted water samples showed that there was an inhibition effect when assayed using the KQCL™ LAL assay as the concentration of the LPS is almost ten times lower than the corrected value of the 1/10 and 1/100 dilutions. To confirm the inhibition incidence, a spike and recovery assessment was performed by spiking a final concentration of 0.5 EU mL⁻¹ in artificial seawater (Instant Ocean, Underworld, UK) with appropriate controls and then run those samples through the LAL assay (Table 3.2). Recovery was calculated in percentage as follows:

$$\text{Recovery\%} = \frac{\text{Analytical}}{\text{Theoretical}} \times 100$$

Table 3.2: Spike and recovery assessment of LPS in Instant Ocean to test for Inhibition/enhancement in performing LAL assay.

Diluent	Spike	Expected	Observed	Recovery%
Instant ocean	0.5 EU mL ⁻¹	0.5 EU mL ⁻¹	0.042 EU mL ⁻¹	8.4%
LAL water	0.5 EU mL ⁻¹	0.5 EU mL ⁻¹	0.5 EU mL ⁻¹	100%
Instant ocean	Negative control	<0.005 EU mL ⁻¹	<0.005 EU mL ⁻¹	Nil
LAL water	Negative control	<0.005 EU mL ⁻¹	<0.005 EU mL ⁻¹	Nil

It seems that salinity of marine water samples has an inhibition effect on the KQCL™ assay and this inhibition effect can be overcome by diluting the sample 1/100 fold.

The LPS levels of the stream (Figure 3.8) showed to be the highest among the other areas, because it was the main contamination source to that particular beach based on the number of FIB, *Bacteroides* species and total coliforms.

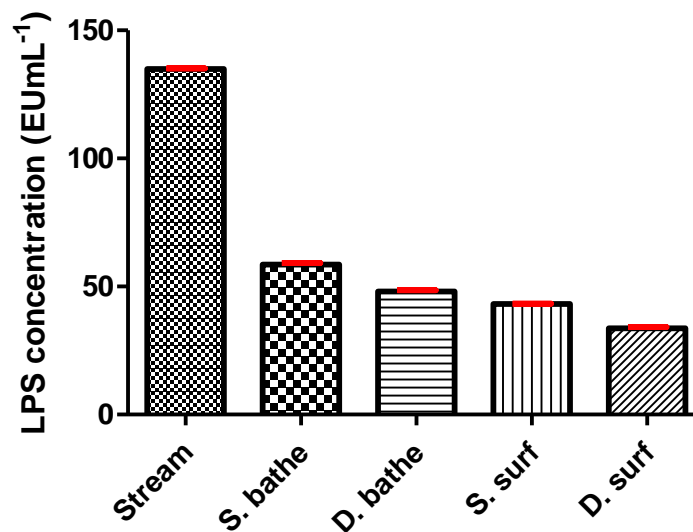


Figure 3.8: Mean LPS concentrations of 10 composite water samples from five areas. A one-way ANOVA and Tukey's Multiple Comparison Test were conducted to evaluate the difference in LPS concentrations among different water sampling areas. There was a significant difference between all areas with $p < 0.001$. Stream = water samples from the stream area, S. bathe = shallow water samples from the bathing area, D. bathe = deep water samples from the bathing area, S. surf = shallow water samples from the surfing area, D. surf = deep water samples collected from the surfing area. $n=4$. Error bars (in red) show standard error of the mean.

Since the S. bathe area was the most affected area of the beach (by the stream) individual water samples from the shallow bathing area were assessed for their LPS activity and plotted with *E. coli* and *Bacteroides* species to see if the LPS levels correspond to the levels of these 2 bacteria (Figure 3.9).

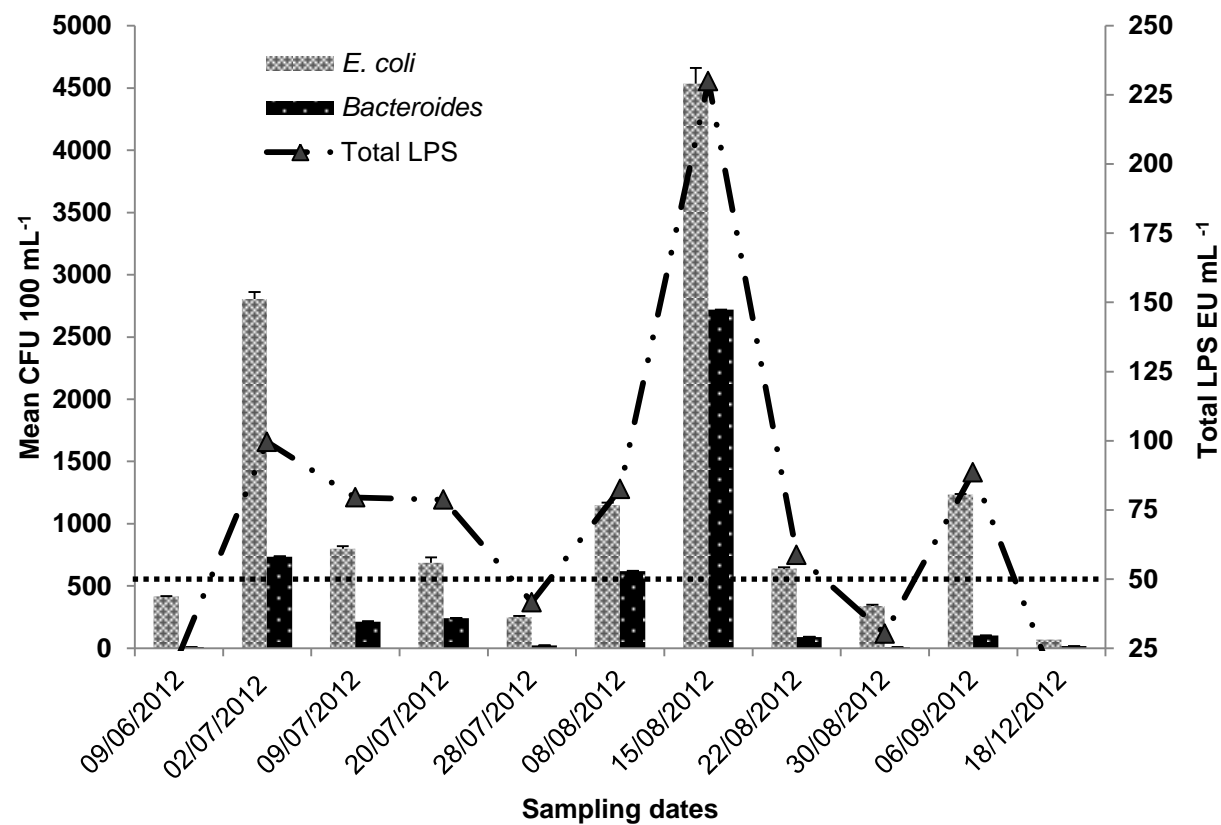


Figure 3.9: Mean number of *Bacteroides* and *E. coli* CFU mL⁻¹ and total LPS concentration EU mL⁻¹ in water samples (n=4) of the shallow bathing area during the sampling periods. The dotted line represents the cut off of LPS concentration above which readings are considered as unsuitable water for bathing.

3.9.3 Correlation between total LPS levels and FIB in marine bathing waters

Good correlations between the number of indicator bacteria and concentrations of total LPS were obtained (Figure 3.10). The European directive threshold value for *E. coli* (500 CFU 100 mL⁻¹) is also shown in figure 3.10 A and is converted to EU mL⁻¹ of LPS.

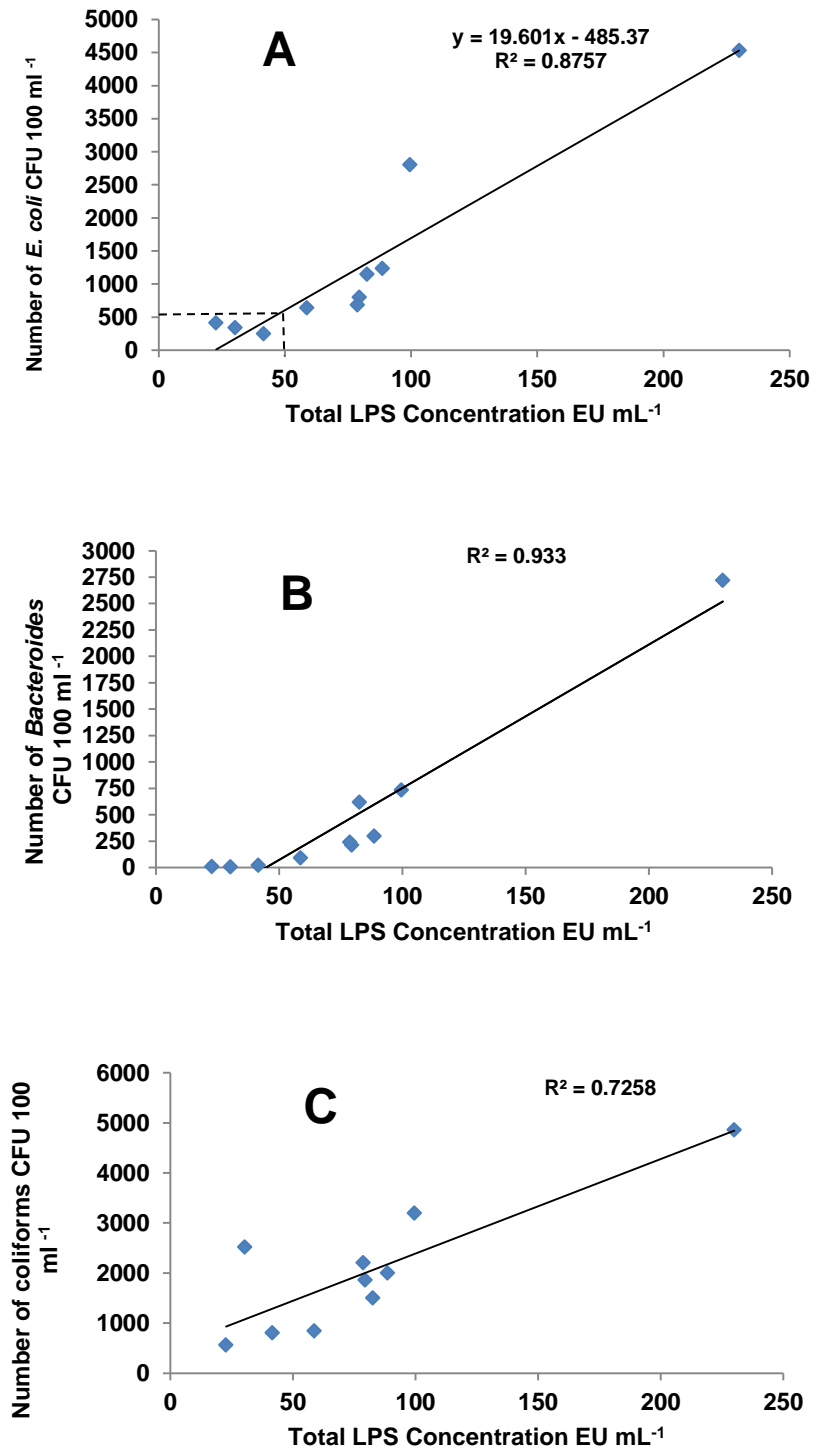


Figure 3.10: Pearson correlation of 10 composite water sample means for CFU and total LPS at the shallow bathing area (S. bathe) (A) *E. coli*, Pearson correlation of LPS and *E. coli* = 0.936, $p < 0.001$ (B) *Bacteroides*, Pearson correlation of LPS and *Bacteroides* = 0.954, $p < 0.001$ and (C) total coliforms, Pearson correlation of LPS and total coliforms = 0.852, $p < 0.05$. A threshold as set by the European directive and the corresponding LPS concentration is highlighted with the dotted line in figure A

3.9.4 Endpoint chromogenic LAL assay optimisation

Optimised endpoint chromogenic LAL assay was successfully tested on Challaborough beach. Samples from the stream, bathing and surfing areas were instantly tested and results were comparable with the enumeration of FIB using culture based method. {Data not shown because this piece of work is patent pending (**UK Patent application - GB1312635.4 Water Testing – filed on 15th July 2013 (Priority Date) by the University of Plymouth**)}.}

3.10 Discussion

Testing the quality of marine bathing waters is an essential procedure to meet the European Union bathing water directive and ensure the health of bathers and watersports performers. However a real-time cost effective method is needed as current culture-based methods are time consuming with retrospective results reflecting the water quality status at least 24-48 hours retrospectively. Results from this chapter have shown that LPS can be potentially used as a rapid, qualitative biomarker of marine faecal contamination. Studies conducted previously have explored the use of LPS as an indicator for testing contamination in drinking and other water. Jorgensen *et al* (1976) ran a pilot study to explore the possibility of using LAL assay to estimate the concentration of LPS in potable and reclaimed advanced waste treatment and showed that chlorination of water interferes with the assay. Watson *et al* (1977) used three techniques including the determination of LPS concentration to estimate the biomass and number of bacteria in marine water; that study

showed that LPS can be related to the number of bacteria and suggested a factor to convert LPS to bacterial carbon. LPS concentration in water supplies from a stream water correlating with the culture-based bacterial count of coliforms, enteric, Gram-negative and heterotrophic bacteria was investigated by Evans *et al* (1978) using the gel clot and a spectrophotometric LAL assay. This showed that the gel clot method was less sensitive and less reproducible than the spectrophotometric assay; Evans *et al* (1978) also suggested to continue refining of the LAL assay for implementation in water quality investigation.

In this chapter, a more accurate and highly sensitive kit was used to investigate the bacteriological LPS activity of recreational bathing seawater. This kinetic KQCL™ LAL assay can detect LPS down to 0.005 EU mL⁻¹. Results showed that total LPS concentration correlates very well with the current bacterial indicator, *E. coli* and with *Bacteroides*, and slightly less with total coliforms (Figure 3.10) all including a pollution event after heavy rainfall. Increases in total LPS levels also appeared to dissipate from the bathing water within the time period of the next sample (7 days), probably within the next 70 hours by the methods described by Shibata (2009). LPS molecules are shed from the Gram-negative bacterial cell wall when the environmental factors are not suitable for these cells or when infected with bacteriophages (Fuhrman, 2000, Nagata, 2000). LPS is being constantly removed from seawater by flagellates (Shibata *et al*, 2009). The LPS threshold at which seawater can be considered as polluted or unsuitable for bathing was determined based on the currently legislated number of CFU for a “Sufficient” level of bacterial indicators in the

European directive 2006 guide for coastal bathing water quality. Using figure 3.9 the author calculated the corresponding LPS concentration at these limits to be equivalent to 50.3 EU mL⁻¹. This level could be set for a future real-time qualitative bathing water test kit development. The salinity of seawater is generally 35 parts per thousand (35 o/oo) in most marine bodies (Pinet, 2009). The salinity is measured by collectively measuring the different salt component dissolved in seawater. Interestingly, dissolved salts always constituted of the same ratio and type of salts even when salinity differs from average seawater. Sodium and Chloride comprises the major two salts in seawater; comprising 85.62% of the total salts. Schleef (1979) and colleagues demonstrated that Sodium Chloride appears to interfere with gel clot LAL assay. In this study, this problem of salt inhibiting the LAL assay has been overcome by diluting seawater samples with LPS-free water.

Marine bathing water represents a sanctuary for eukaryotes and prokaryotes including indigenous Gram-negative bacteria. When estimating the total LPS from marine bathing waters, part of the total LPS measurement is due to the presence of background indigenous Gram-negative bacteria which will affect the estimation of LPS using the LAL assay. However, many studies have shown that the major bacterial species present in seawater are characterised by low LPS potency because of low acylation and phosphorylation is encountered in marine Gram-negative bacteria (Krasikova *et al.*, 2004, Leone *et al.*, 2007, Ramos *et al.*, 2001). The presence of low acylation, penta and tetra acyl species, and phosphorylation in the lipid A structure signifies low biological activity compared to endotoxically active molecules (Rietschel *et al.*, 1994) and

also an indication of low activity with the LAL assay (Takada *et al.*, 1988). Hence, even when there is an increase in the background levels of Gram negative bacteria; it is likely to induce a low reactivity in the LAL assay. Collected water samples processed in this study have never shown false positive results in the LAL assay, and low CFU numbers of FIB (especially *E. coli*) were always accompanied with low LPS activity in the LAL assay. However, there is a possibility of occasionally observing false positives (events above 50 EU mL⁻¹) in the absence or low numbers of FIB, this may perhaps be true due to for example eutrophication (Nixon, 1995) in seawater that allows marine Gram-negative bacteria to thrive. A less likely possible source is from cyanobacterial blooms and an increase in *Vibrio* densities. Although these increases in total LPS would be related to a non-faecal pollution event, this can still be useful as an indicator for the presence of these blooms which have been shown to cause serious human health problems such as gastrointestinal tract infections, cyanobacterial intoxications and even cholera (Morris and Acheson, 2003, Dietrich *et al.*, 2008). The quantitative KQCL™ LAL assay was performed in approximately one and a half hours when using a ten-fold range of LPS standards 0.005-50 EU mL⁻¹ and approximately 23 minutes to detect 0.5 EU mL⁻¹ and above, which is equivalent to 50 EU mL⁻¹ after 100 fold dilution. An endpoint chromogenic LAL assay (Genscript, USA) was successfully optimised and *in situ* tested the quality of marine bathing water. This assay could be optimised for untrained bathers as a real-time, qualitative, single-use kit to examine the bacterial quality of bathing waters. The LAL method is not an attempt to replace the applicability of current culture based or future PCR

methods as these are a valuable method used in microbial source tracking but could be used as part of a “tool box” approach to water quality management.

Whilst background levels of endogenous LPS appeared to remain low and stable during this study a *specific* LPS assay for one indicator would be preferable. The use of *Bacteroides* LPS has a high potential to be used as a real-time, specific biomarker. The abundance of *Bacteroides* in faeces is an advantage as it can serve as a specific indicator for recent faecal pollution in marine bathing waters. *Bacteroides* species are strictly anaerobic bacteria; their inability to reproduce in seawater excludes the possibility of false overestimation as occasionally occurs with *E. coli* and enterococci. Detailed information regarding the use of *Bacteroides* LPS as a biomarker is discussed in chapter four.

Several studies have looked at the background levels of LPS in the environment, these studies focused mainly on sampling air in composting sites, wastewater plants and others. LPS were usually expressed either in EU/m³ or ng/m³. Researchers have attempted to establish a health based occupational and environmental exposure threshold. The Dutch Expert Committee on Occupational Standards (DECOS) set a value of 50 EU/m³ as an occupational exposure threshold level for LPS (DECOS, 1998) which then was altered to 200 EU/m³ based the potential inhalable dust exposure in an 8 hours working day. Although the LPS level suggested by DECOS was not regarded in other countries, several studies conducted in different working environments recorded 1-1000 fold higher incidents (Liesivuori *et al.*, 1994, Milton *et al.*, 1995, Simpson *et al.*, 1999). A study conducted by Ginger *et al* (2006) measuring mould and

LPS levels after Hurricane Katrina in the USA estimated that LPS background levels in the environment ranged from 17 to 139 EU/m³ air sampling. Lee *et al* (2006) conducted a study to assess the air quality of wastewater treatment plants and found out that LPS levels ranged from 6-1247 EU/m³.

Previous studies have discussed the possible health effects of airborne dispersal of bioaerosols including LPS in composting sites on employee and neighbouring residents (Liu *et al.*, 2011, Palchak *et al.*, 1988, Pankhurst *et al.*, 2011), however, none has looked at the background level in marine bathing waters. Results here showed that the total LPS levels in “Excellent” water samples showed a very low number of FIB and the LPS level was as low as 6 EU mL⁻¹, hence 6 EU mL⁻¹ was suggested as a background level of total LPS in the study area. The levels of total LPS might vary from one beach to another depending on various factors such as nutrition (Nixon, 1995), temperature, UV levels (Anderson *et al.*, 2003). Hence, different beaches need to be evaluated in terms of background LPS levels which could be explored in the future work.

**Chapter 4: The potential of *Bacteroides* species LPS as
a specific faecal biomarker in marine bathing waters.**

4.1 Introduction

The potential of total LPS as a real time biomarker has been investigated in chapter three. Despite the fact that false positive results not once occurred during this study, it has been hypothesised that they might occur. Therefore, a more specific method that could be used as a real time biomarker for faecal contamination is required. Hence, LPS of *Bacteroides* species was chosen. Before explaining why *Bacteroides* LPS in particular was chosen, a few main points of *Bacteroides* advantages over current European Union bathing Directive faecal indicator bacteria needs to be addressed. It has been suggested by previous studies that there are several drawbacks in using faecal indicator bacteria not only because results are retrospective taking 24-48 hours; but also because *E. coli* and enterococci are capable of survival and proliferation in the environment (Ahmed *et al.*, 2008). *Bacteroides* species represent an excellent candidate to be considered as indicators for more recent faecal pollution (Fiksdal *et al.*, 1985, Kildare *et al.*, 2007, Kreader, 1995) and are predominant components of the normal flora of the mucosal membrane in human intestines being even more predominant than *E. coli* in faeces (Fiksdal *et al.*, 1985). Furthermore, *Bacteroides* species are incapable of replicating outside their host, i.e. human and warm blooded animals, and thus a more representative indicator for faecal contamination than *E. coli*; however, because of their strict anaerobic nature, they are more difficult to culture. Clinically, *Bacteroides fragilis* is one of the most important anaerobic Gram-negative rod (Gorbach and Bartlett, 1974) because it is opportunistic and it is one of the causative agents affecting human health such as abdominal abscess,

appendicitis, female genital tract abscesses in addition to other types of infections (Joseph and Julian, 1970, Norwalk and Appleton, 1995, Ryan, 2004). LPS of *Bacteroides* is substantially different to other Gram-negative bacteria (Hofstad, 1975, Kasper, 1976) lacking the smooth complex O side-chain and lacking two components: 2-Keto-3deoxyoctulosonic acid and L-glycero-D-mannoheptose. Unlike coliforms which might have a consistent background level in seawater, *Bacteroides* levels increase during faecal pollution events from around zero to 2700 CFU 100 mL⁻¹ (Chapter 3), thus increasing the chance of exposing bathers to the *Bacteroides* lipopolysaccharide during faecal pollution and making the *Bacteroides* LPS a promising candidate for a potential biomarker. On the other hand, *Bacteroides* play an important role as normal flora which compete with other potentially pathogenic bacterial species and prevent them from colonising the intestinal tract since *Bacteroides* is the most abundant bacterium in human gut (Norwalk and Appleton, 1995). Since *Bacteroides* species represent almost a quarter of the intestinal microbiota in the human intestine, and if a recent faecal or sewage pollution takes place in bathing waters, this might have a potential human health risk for persons drinking, swimming/bathing and eating shellfish harvested from water that contains *Bacteroides* species as well as pathogens.

Because current culture based methods are retrospective, and results in chapter three suggest the use of LPS as a biomarker, a more faecal-specific indicator is required to avoid any possible false positive estimation of total LPS using the LAL assay. Hence, LPS of *Bacteroides* species was chosen as a biomarker indicator of faecal pollution in marine bathing waters.

The objectives of this chapter were thus to enumerate and identify *Bacteroides* species present in local bathing seawater using phenotypic and genotypic methods to investigate the origin of this faecal source using genomic microbial source tracking (microbial source tracking), followed by extraction of the *Bacteroides* LPS. Extracted LPS was then used to:

- Assess the activity of LPS from *Bacteroides* species using the LAL assay to confirm the potency and explore the percentage of LPS activity contribution to the total LPS activity in marine bathing water.
- Profile purified LPS from *Bacteroides* species for similarity in banding pattern.
- Investigate the biological activity in cell culture models to examine the potential biological activity by examining the inflammatory response of *Bacteroides* LPS on human monocytic Mono Mac 6 cell line and mouse MPI macrophages by measuring three main proinflammatory cytokines TNF- α , IL-6 and IL-1 α .
- Design a method to detect the *Bacteroides* LPS using ELISA assay as a more specific faecal biomarker in marine bathing waters.

4.2 Materials and methods

4.2.1 Enumeration of *Bacteroides*

Marine bathing water samples were filtered using the methods described in chapter three (section 3.2.2). Briefly, *Bacteroides* isolates were enumerated using classical culture based methods and expressed in colony forming units

per 100 mL of bathing water as described in the materials and methods in chapter three. Randomly, 101 colony forming units were selected for further speciation and downstream work.

4.2.2 Phenotypic and genotypic methods for the characterisation of *Bacteroides* species isolated from bathing water

Traditionally, biochemical testing and speciating of bacteria plays a key role in identifying unknown bacterial isolates based on phenotypic, biochemical and sugar utilisation tests. Although there are recent advancements in molecular methods in the field of microbiology regarding the identification of bacteria, performing biochemical tests is still an essential routine practice to be performed in the field of microbiology. Classical biochemical speciation of *Bacteroides* was performed on 101 isolates to identify different *Bacteroides* species present in marine bathing water.

4.2.3 Growing *Bacteroides* isolates on BBE agar and esculin hydrolysis

One of the first steps in the presumptive identification of *Bacteroides* species is their ability to grow on the *Bacteroides* bile Esculin medium (BBE) (Livingston *et al.*, 1978). *Bacteroides* Bile Esculin agar is an enriched, selective and differential medium for the isolation and primary identification of *Bacteroides* species. Firstly, BBE contains gentamicin at a concentration which inhibits most

facultative anaerobes except *Bacteroides* species. Secondly, BBE medium also contains bile salts, an inhibitor of Gram-positive and anaerobic Gram-negative rods inhibitor except for *Bacteroides* species. Esculin hydrolysis characterised by a brown to black colouration in the medium around the bacterial colonies is another feature of *Bacteroides* identification. A good primary identification of *Bacteroides* was achieved by initially growing them on the BBE agar.

4.2.4 Obtaining pure cultures of *Bacteroides* isolates

After colony development of *Bacteroides* isolates on BBE agar; *Bacteroides* isolates were randomly selected and each colony was inoculated on a freshly prepared BBE agar plate to ensure obtaining pure cultures. Eventually, 101 pure culture of *Bacteroides* isolated were obtained and further investigated for speciation.

4.2.5 Gram stain reaction

A routine Gram staining reaction was conducted initially to all *Bacteroides* isolates to confirm their Gram stain reaction.

4.2.6 Classical *Bacteroides* speciation using biochemical tests

Bacteroides isolates were speciated using biochemical methods described in Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 1994).

4.2.7 Indole test

Indole and sugar utilisation tests were used to differentiate among the *Bacteroides* isolates. In this study, indole and sugar utilisation tests were used as an eliminating method to group 101 *Bacteroides* isolates into groups according to their reaction then confirmed using genotypic methods.

The indole test is a biochemical test that examines the ability of bacteria to break down the amino acid tryptophan into indole. Detection of indole relies upon the chemical reaction between indole and Ehrlich's reagent (Faddin, 2000). Ehrlich's reagent is a modified version from the original Kovac's reagent, prepared as illustrated in table 4.1.

Table 4.1: Ehrlich's reagent ingredients.

Ingredients	Amount
Ethyl alcohol (absolute)	95.0 mL
p-dimethylaminobenzaldehyde (DMAB)	1.0 g
HCl (concentrated)	20.0 mL

Ingredients were dissolved and the solution was stored in a brown glass bottle protected from direct light at room temperature. All 101 *Bacteroides* pure culture isolates were inoculated into *Bacteroides* phage recovery broth medium (BPRM) in order to perform further bacteriological investigations. The Indole test was performed on freshly subcultured pure cultures of *Bacteroides* by taking 0.5 mL of *Bacteroides* broth culture and adding a few drops of Ehrlich's reagent. Development of a red-violet colour in the reagent layer indicates a positive

indole test while negative indole test is indicated when no colour change takes place.

4.2.8 Sugar utilisation tests

Certain *Bacteroides* species are capable of utilising one or more of certain sugars (Bergey and Holt, 1994). In order to identify and speciate *Bacteroides* isolates using sugar utilization method, three sugar types were used: Rhamnose, Trehalose and Salicin as a carbon source instead of glucose which is usually added in *Bacteroides* phage recovery broth medium. Each bacterial isolate was inoculated into 3 different broths supplied with one of the sugars using a 48 well plate. 0.5 mL of broth was added with one of the three sugars with pH indicator (bromophenol blue), as an indicator of bacterial growth and acid production as a side product from sugar utilisation. A colour change in the medium from violate to yellowish colour (Figure 4.1) is regarded as a positive result while no colour change was regarded as a negative result. Results then compared to differential characteristics of the *Bacteroides* species table that was published in Bergey's manual of determinative bacteriology (Bergey and Holt, 1994) and grouped for further molecular identification.



Figure 4.1: Sugar utilisation test for *Bacteroides* speciation. *Bacteroides* isolates were grown in BPRM supplemented with one of the three sugars rhamnose, trehalose and salicin. A: positive sugar utilisation, B: negative sugar utilisation with no colour change.

4.2.9 Speciation confirmation of *Bacteroides* using molecular methods

4.2.9.1 Molecular methods for identification and speciation of *Bacteroides* isolates

Polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene which is highly conserved in Bacteria and Archaea (Coenye and Vandamme, 2003) leading to constructing of genetic library that can be used to compare new sequences to the existing sequences in the database.

4.2.9.2 Genomic DNA extraction from *Bacteroides* isolates

Genomic DNA was extracted from phenotypically speciated pure culture *Bacteroides* isolates using phenol-chloroform-isoamyl alcohol and heating method (Sepp *et al.*, 1994). Briefly, one mL of broth culture was aseptically

taken into a 1.5 mL sterilised microcentrifuge tube and pelleted by centrifugation in a microcentrifuge at 13,000 x g for 10 minutes. The bacterial pellet was resuspended in sterilised phosphate buffer saline pH 7.3 (PBS) as a washing step from any medium remnant. Sample was repelleted by centrifugation in a microcentrifuge at 13,000 x g for 10 minutes then the supernatant was discarded. 500 µL of Tris Low EDTA (TLE: 10 mM tris base pH 8.0, 0.1 mM EDTA) buffer was then added to the bacterial pellet and mixed until homogenised. Samples were then heated in a heat block for 10 minutes at 100 °C. Samples were pelleted by centrifugation at 13,000 x g for 10 minutes, and then supernatant was transferred into a new sterile microcentrifuge tubes. An equal volume of a pre-mixed phenol-chloroform-Isoamyl alcohol (25:24:1 ratio respectively) was added to each tube, briefly mixed and then pelleted by centrifugation at 13,000 x g for 10 minutes. The supernatant was then aspirated into a new sterilised microcentrifuge tubes to assess quality and quantity of extracted genomic DNA. The quality (A260/A230 ratio for residual phenol contamination, A260/A280 ratio indicate protein contamination) and the concentration of extracted DNA were determined using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific Ltd., DE, USA).

4.2.9.3 Polymerase Chain Reaction (PCR) conditions

PCR amplification of extracted genomic DNA was carried out using universal *Bacteroides* primers, forward primer Bac32F 5'- AACGCTAGCTACAGGCTT-3' (Bernhard and Field, 2000a) coupled with the reverse primer 5'- Bac708R CAATCGGAGTTCTTCGTG-3' primer set manufactured by Eurofins (MWG

BiotechLtd., Germany). Each PCR tube contained 1 μL of both forward and reverse primer (50 pmol L^{-1}) 1 μL of DNA template, 12.5 μL red TaqTM PCR reaction mix (Bioline, UK) and 9.5 μL of DNA grade water.

The PCR conditions were as follows (Thermocycler: Techne tc-512 PCR geneglow U.K), 35 cycles comprising of 94 °C for 30 seconds, 53 °C for 1 minutes, and 72 °C for 2 minutes, followed by a final 6 minutes extension at 72 °C (Bernhard and Field, 2000b). PCR products were run on agarose gels to assess PCR success.

4.2.9.4 PCR condition for sequencing using 27f and 1492r primer set.

Amplification of the 16s rRNA gene for sequencing was carried out using 27F 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R 5'-ACCTTGTTACGACTT-3' primer set. 35 cycles at 94 °C for 2 minutes, 42 °C for 30 seconds, and 72 °C for 4 minutes; followed by a final extension period of 20 minutes at 72 °C (Lane, 1991).

4.2.9.5 Agarose gel electrophoresis and gel imaging

1.5% agarose gels were prepared by weighing 1.5 g of agarose (Sigma-Aldrich, UK) and added into a final volume of 100 mL of Tris-acetate-EDTA (TAE) buffer (10X TAE Buffer: 48.4 g Trizma base + 20 mL of 0.5M EDTA pH 8.0 + 11.44 mL glacial acetic acid). The suspension was heated to boiling point in a microwave and then placed in a 56 °C water bath to cool. Eight μL of SYBR[®] Safe DNA gel

stain (Invitrogen, U.K.) was added just before casting the gel into a gel casting tray (Alpha Laboratories electrophoresis tank, U.K) and left to solidify at room temperature. Ten μL of PCR amplicon were carefully loaded into the agarose gel well alongside a 50 bp DNA ladder (Sigma Aldrich, U.K). The gel was run at 80 volts for 30 minutes and then imaged using a Gel Doc XR System Bio-Rad scanning and Quantity One[®] software (Bio-Rad).

4.2.9.6 Post-PCR clean-up for DNA sequencing

Samples were prepared for sequencing using Sanger sequencing method (Sanger *et al.*, 1977). PCR products were cleaned up using microCLEAN (Microzone, UK) DNA clean-up reagent to remove any residue of dNTPS, primers and polymerase from PCR products before sending for sequencing. PCR products were cleaned according to the manufacturer's instructions, briefly, In a 1.5 sterile microcentrifuge tubes, an equal volume of microCLEAN was added to DNA sample, mixed by pipetting then left for 5 minutes at room temperature. Tubes were then spun at 13,000 x g in a micro centrifuge for 7 minutes, supernatant was discarded then another spin step for 2 minutes was performed. Remnant reagent was carefully removed and the DNA pellet was resuspended with 30 μL of DNA grade H_2O (Sigma, Aldrich, UK). DNA was left for 5 minutes to rehydrate at room temperature then estimated using the Nano drop and diluted as needed. 5 μL of purified PCR product between 20-80 $\text{ng } \mu\text{L}^{-1}$ was mixed with 5 μL of 5 $\text{pmol } \mu\text{L}^{-1}$ of reverse primer R1429 then sent as instructed to GATC Biotech sequencing company (GATC, Germany).

4.2.9.7 16S rRNA Gene sequencing for *Bacteroides* identification

DNA sequencing is a method of determining the exact order of any of the four nucleotide bases adenine, guanine, cytosine, and thymine within a DNA segment which is an essential approach to identify the species and the origin of that DNA strand. It is well established that the 16S rRNA gene is highly conserved in bacteria and can be considered as a unique fingerprint for each bacterium. DNA sequencing was used in this study to confirm the speciation of *Bacteroides* species isolated from bathing water samples using Sanger sequencing technique. Sequences were analysed using MEGA5 version 5.1 software (Tamura *et al.*, 2011).

4.2.9.8 Comparing sequences against BLAST database

Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) is a revolutionary tool in the field of molecular biology used to compare the similarity between sequenced nucleotides or proteins with an already established gene databases library (Madden, 2002). This tool was used in this study to investigate the similarity and confirm bacterial species identification. Automated DNA sequencers produce a four-color chromatogram (A=green, C=blue, G=black and T=red) each colour represents 1 nucleotide showing the results of the sequencing run. Chromatograms were viewed using MEGA5 and manually checked to assess the quality of the sequencing output for any artefacts or errors such as miss-spaced or double heterozygous peaks. These irregular peaks could be due to primer interference and background noise and generally

loss of resolution take place later in the gel (Figure 4.2 A). Trimming irregularities and artefacts that usually appear at the beginning and the end of a sequence was achieved by selecting these areas using the mouse computer or keyboarded (CTRL+ right or left arrow) then deleted using the delete key on keyboard (Figure 4.2 B), the trimmed sequence was then exported as a FASTA file and then compared to BLAST search database by clicking on the highlighted icon with the black circle in figure 4.2.

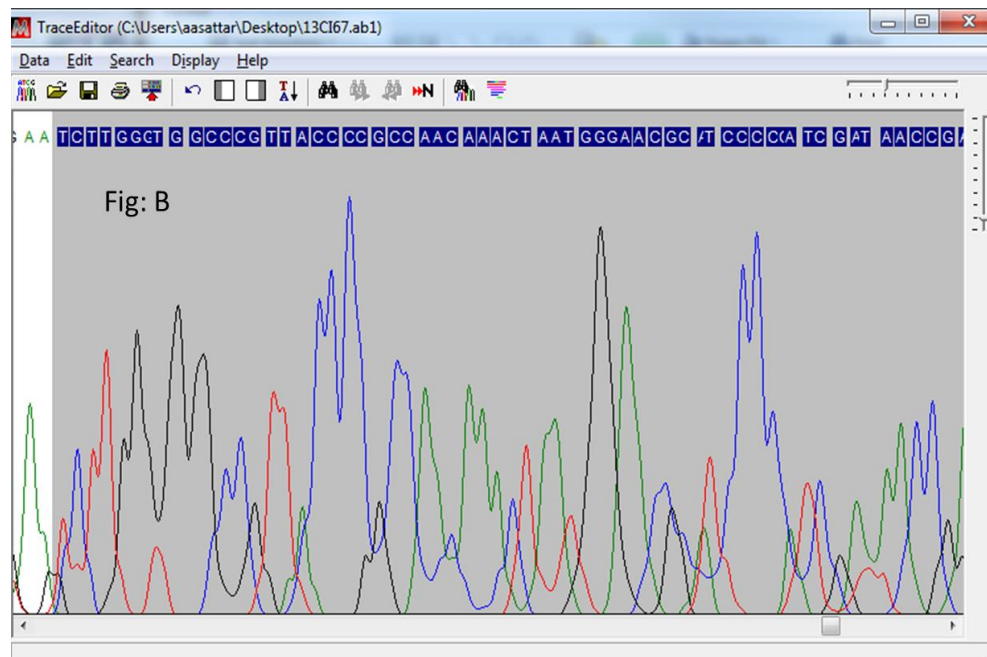
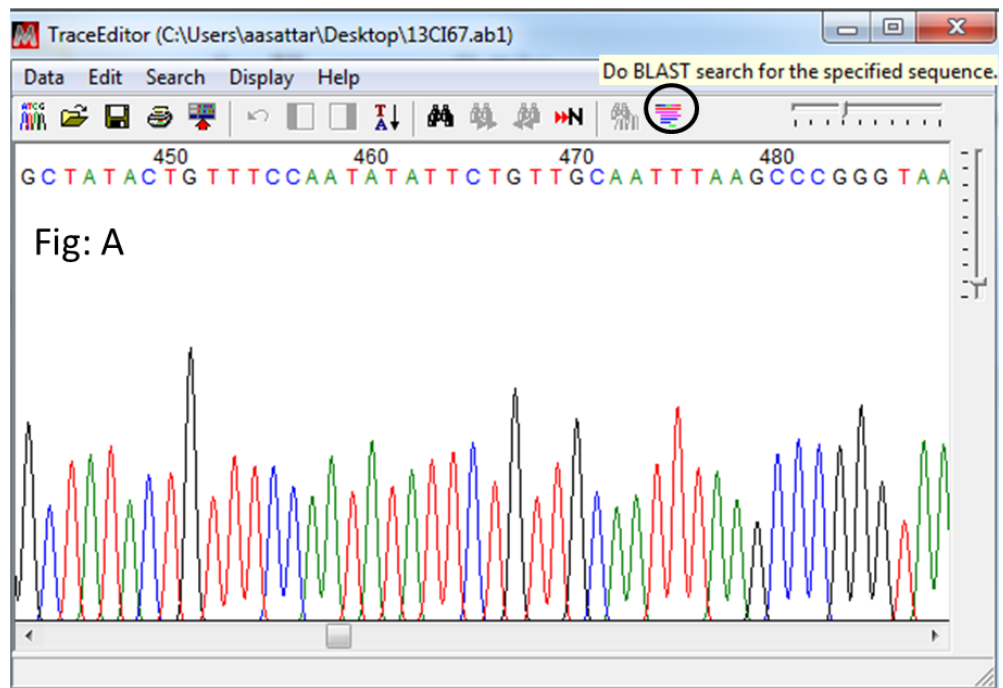


Figure 4.2: Two Genomic DNA Sequence chromatograms of the 16S rRNA gene of one of the isolated *Bacteroides* viewed with Mega5 software. Fig (A) shows evenly-spaced peaks and the lack of baseline 'noise' while Fig (B) shows overlapped peaks towards the end of a sequence; these peaks were selected (grey area) as described above and deleted.

After clicking the blast icon, a MEGA5 web explorer window opens NCBI blast search webpage (Figure 4.3).

http://www.ncbi.nlm.nih.gov/blast/blast.cgi?cmd=web&layout=onewindows&auto_format=fullauto&page=nucleotides&ncbi_gi=yes&filter=l&hitlist_size=100&show_overview=yes&auto_format=yes&show_linkout=yes.

Appropriate options including the 16S rRNA sequencing (Bacteria and Archaea) and highly similar sequences were selected as illustrated in figure 4.3 then BLAST button at the lower left corner of the page was clicked. Results usually appear in approximately 20-30 seconds.

M5: Web Browser (Nucleotide BLAST: Search nucleotide databases using a nucleotide query)

Data Edit View Links Go Help

Address: [https://blast.ncbi.nlm.nih.gov/Blast.cgi?GI=yes&FILTER=L&HITLIST_SIZE=100&SHOW_OVERVIEW=yes&AUTO_FORMAT=yes&SHOW_LINKOUT=yes](#)

Links: NCBI

BLAST® Basic Local Alignment Search Tool

Home Recent Results Saved Strategies Help

NCBI/ BLAST/ blastn suite **Standard Nucleotide BLAST**

blastn blastp blastx tblastn tblastx

BLASTN programs search nucleotide databases using a nucleotide query. [more...](#)

Enter Query Sequence

Enter accession number(s), gi(s), or FASTA sequence(s) [Clear](#) [Query](#)

GATCTGATTACACTAAGAGCTCTTGCAGTTACGTACTTCAGGTACCCCGGCTTTCATGGCTTGA
CGGGCGGTGTGTACAAGCCCGGGAACGTATTCACCGCGCCATGGCTGATGCGCGATTACTAGCG
AATCCAGCTTCACGAAGTCGGGTTGCAGACTTCGATCCGAAGTGAAGAGGTTTTTGGGATTAGC
ATCCTGTCACAGGTAGCCGCTTCTGTACCCCATTTGTAACACGTGTAGCCCGGACGTAA
GGCCGCTGCTGATTTGACGTATCCCACTTCCTCACATCTTACGACGGCAGTCTCTCTAGAGT

Or, upload file [Browse...](#)

Job Title

Enter a descriptive title for your BLAST search

☐ Align two or more sequences

Choose Search Set

Database ☐ Human genomic + transcript ☐ Mouse genomic + transcript ☐ Others (nr etc.):

16S ribosomal RNA sequences (Bacteria and Archaea)

Organism Optional

Enter organism name or id—completions will be suggested ☐ Exclude +

Enter organism common name, binomial, or tax id. Only 20 top taxa will be shown.

Exclude Optional

☐ Models (XM/XP) ☒ Uncultured/environmental sample sequences

Entrez Query Optional

Enter an Entrez query to limit search

Program Selection

Optimize for

☒ Highly similar sequences (megablast)

☐ More dissimilar sequences (discontiguous megablast)

☐ Somewhat similar sequences (blastn)

Choose a BLAST algorithm

BLAST Search database 16S ribosomal RNA sequences (Bacteria and Archaea) using Meg highly similar sequences

Figure 4.3: A standard nucleotide BLAST webpage from NCBI, trimmed sequencing chromatographs were run through the blast search as it shows the nucleotide sequence in this figure, 16S rRNA option was selected from a drop menu and the uncultured/environmental samples sequences were excluded by ticking the box to the left and then the equerry search was optimised to conduct the run for highly similar sequences and then BLAST button at the left lower corner was clicked to start the blast search.

DNA sequences were compared to the GenBank library and a similar page to figure 4.4 appeared showing description of similar sequences organisms' name, maximum score, query coverage, E value, maximum identity and the accession number. These parameters are important to determine which sequences needed to be included in the phylogenetic tree. Highly similar sequences were added to the alignment library in MEGA5 for further phylogenetic tree analysis.

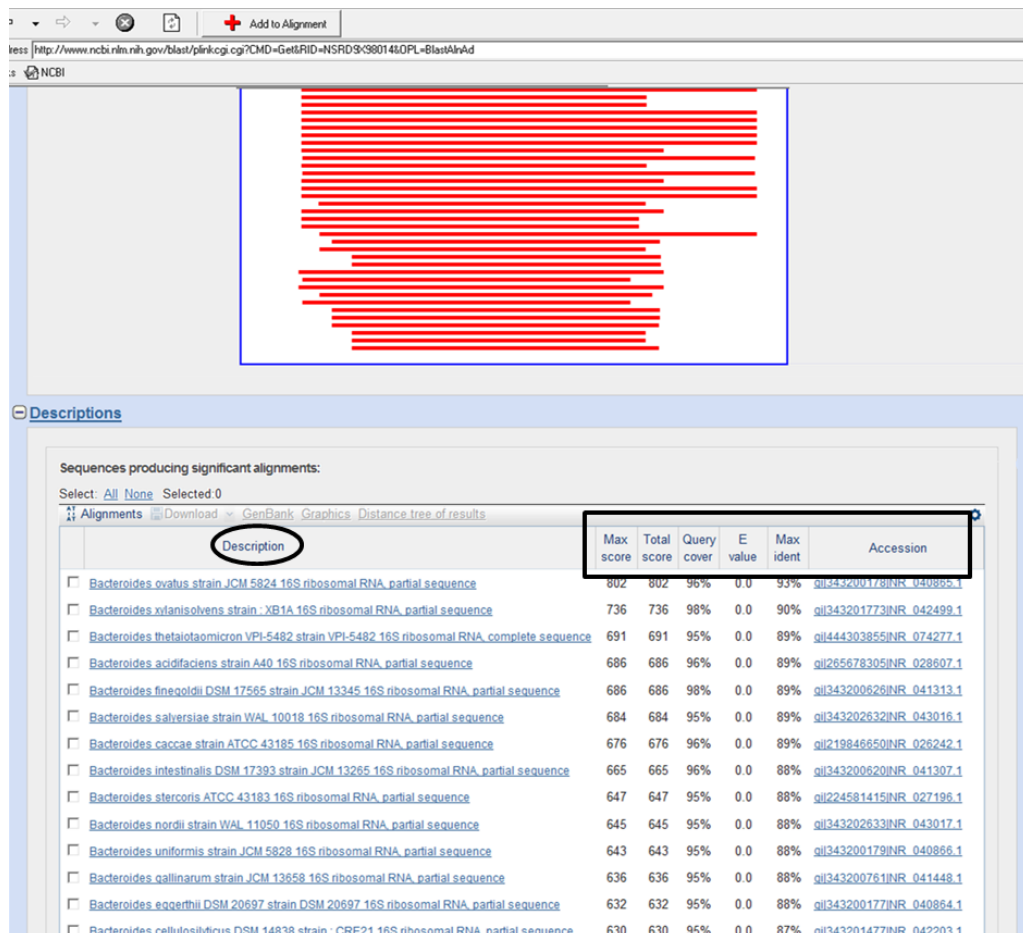


Figure 4.4: an example of BLAST search results showing sequences giving significant alignments to the query sequence depending on the values of highlighted parameters where to the left is “Description” that provide a brief information about the name of the organism, the region of amplification and the strain identity for type culture strains, “Max score” is the score for the highest scoring segment of the subject sequence, total score is the sum of the scores for all segments of the subject that align, “E value” or expected value is the number of hits that one would encounter by chance with the observed score, while “Max identity” represents the percentage similarity between query sequence and the segment of the subject sequence that that is most identical to the query. Accession numbers are unique numbers that identifies a particular sequence in GenBank.

4.2.9.9 16s rRNA gene sequencing submission to GenBank

In order to submit sequences to GenBank, sequences were compared to BLAST search library for the 16s rRNA similarity as shown in figure 4.5.

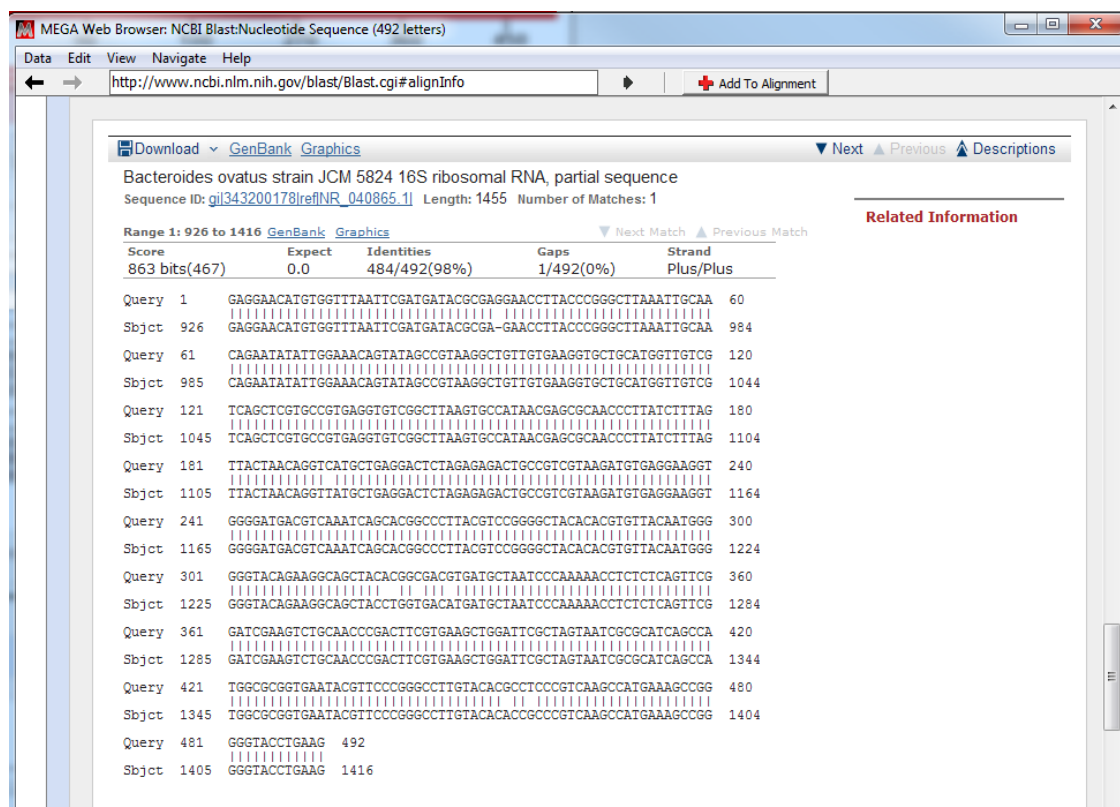



Figure 4.5: A screen shot of BLAST search result showing alignment between query sequence and subject sequence.

Sequences were submitted to GenBank using BankIt tool as explained in the NCBI website: (<http://www.ncbi.nlm.nih.gov/genbank/submit/>). Sequences were given a unique accession number and are shown in the results section.

4.2.9.10 Phylogenetic analysis and Constructing *Bacteroides* species phylogenetic tree

Phylogenetic tree was constructed after adding highly similar sequences in addition to the query sequences. Multiple DNA sequences were aligned using ClustalW tool implemented in Mega 5.1 software in order to run maximum likelihood phylogenetic tree. DNA alignment is an essential step that introduces gaps into the sequence to shift nucleotides back to their original homologues position. Highly similar sequences were added to be aligned by clicking on their accession numbers which will open a new MEGA5 webpage containing the sequence of that accession number and information of the authors and a link to paper publications if it was published. These sequences can be added easily to the MEGA5 alignment explorer by clicking on the “Add to alignment button” as illustrated in figure 4.6

ribosomal RNA, partial

nk=1&RID=NUVGYSYB01R  [+ Add To Alignment](#)

NCBI Resources ☒ How To ☒

Nucleotide [Limits](#) [Advanced](#)

[Display Settings:](#) ☒ GenBank [Send to:](#) ☒

Bacteroides ovatus strain JCM 5824 16S ribosomal RNA, partial sequence

NCBI Reference Sequence: NR_040865.1

[FASTA](#) [Graphics](#)

[Go to:](#) ☒

LOCUS	NR_040865	1455 bp	rRNA	linear	BCT 10-AUG-2011
DEFINITION	Bacteroides ovatus strain JCM 5824 16S ribosomal RNA, partial sequence.				
ACCESSION	NR_040865				
VERSION	NR_040865.1 GI:343200178				
DBLINK	Project: 33175 BioProject: PRJNA33175				
KEYWORDS	.				
SOURCE	Bacteroides ovatus				
ORGANISM	Bacteroides ovatus Bacteria; Bacteroidetes; Bacteroidia; Bacteroidales; Bacteroidaceae; Bacteroides.				
REFERENCE	1				
AUTHORS	Miyamoto,Y., Watanabe,K., Tanaka,R. and Itoh,K.				
TITLE	Rapid Identification of Human Intestinal Bacteroides by 16S rDNA-targeted Species-specific Primers				
JOURNAL	Unpublished				
REFERENCE	2 (bases 1 to 1455)				
CONSRIM	NCBI RefSeq Targeted Loci Project				
TITLE	Direct Submission				
JOURNAL	Submitted (08-AUG-2011) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA				
REFERENCE	3 (bases 1 to 1455)				
AUTHORS	Miyamoto,Y.				
TITLE	Direct Submission				
JOURNAL	Submitted (17-OCT-2000) Yukiko Miyamoto, Yakult Central Institute for Microbiological Research; Yaho 1796, Kunitachi-shi, Tokyo 186-8650, Japan (E-mail:yukiko-miyamoto@yakult.co.jp, Tel:81-42-577-8962, Fax:81-42-577-3020)				
COMMENT	REVIEWED REFSEQ : This record has been curated by NCBI staff. The reference sequence is identical to AB050108 . This record has been curated by collaboration with an international				

Figure 4.6: A screen shot of *Bacteroides ovatus* 16s rRNA gene accession number webpage from NCBI, similar sequences were added to the MEGA5 alignment explorer by clicking the highlighted top middle button “Add to Alignment”.

After adding sequences to the alignment explorer, DNA sequences were aligned using the ClustalW DNA alignment tool which is symbolised with the highlighted letter W (Figure 4.7).

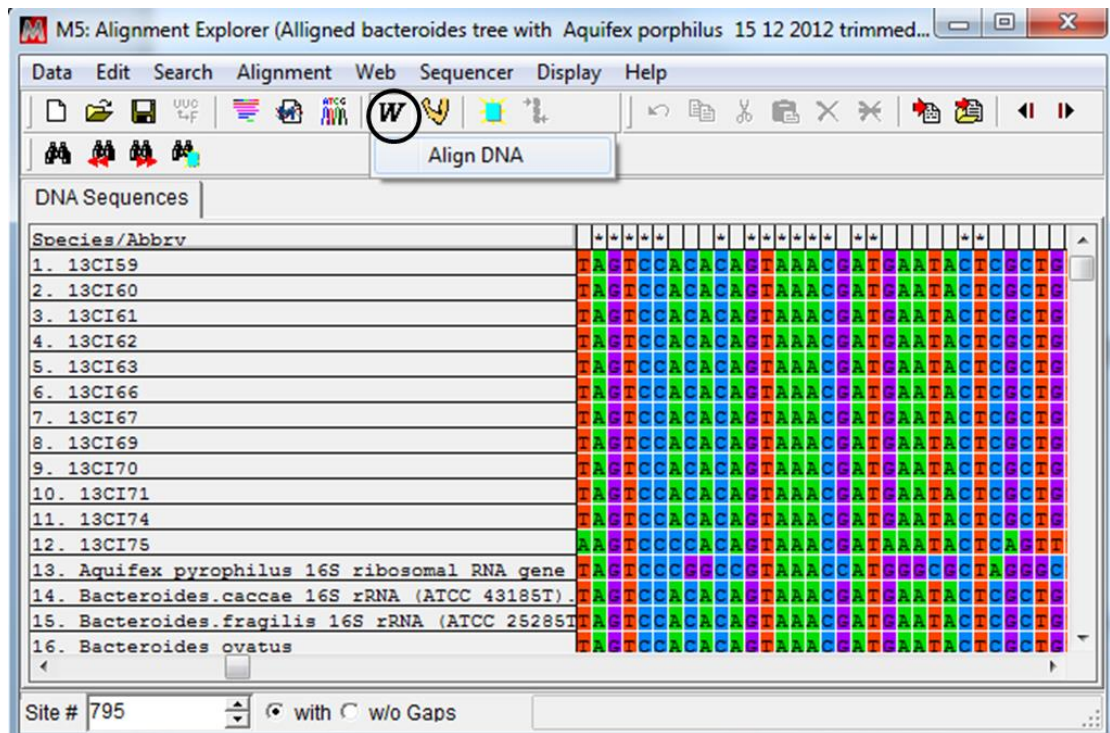


Figure 4.7: A screenshot of MEGA5 alignment explorer page showing DNA sequences of different bacterial sequences (coloured letters) to the right and species name/identity to the left. DNA sequences were aligned by clicking the **W** symbol.

A set of options appears to adjust ClustalW parameter, default recommended (Hall, 2011) settings were used to align DNA sequences as shown in figures 4.8 and 4.9.

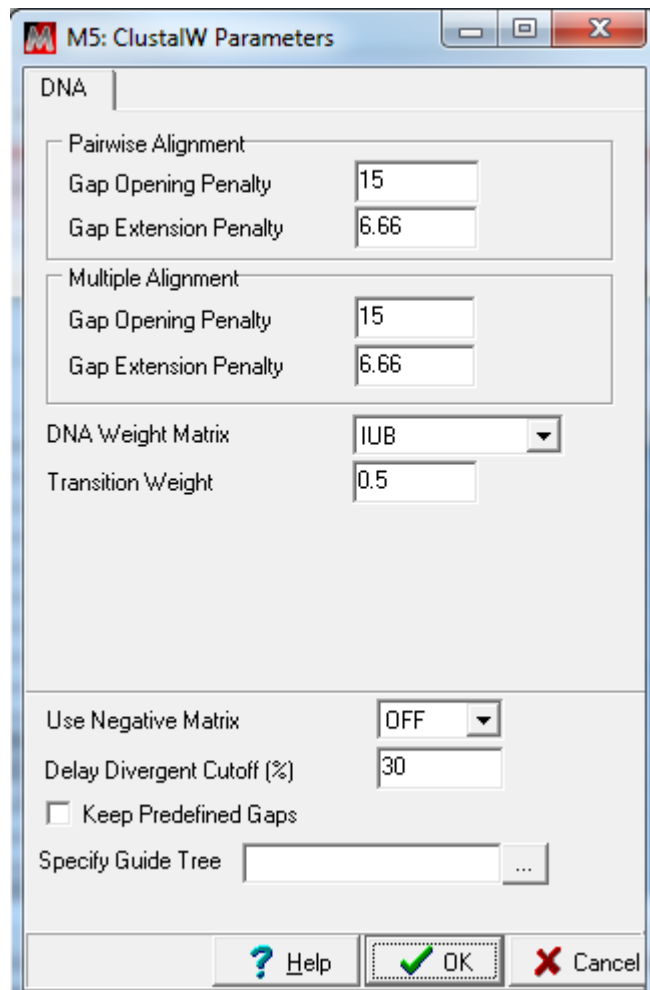


Figure 4.8: A screenshot of MEGA5 showing ClustalW parameters for DNA sequencing alignment.

Pressing the OK button starts the alignment process which can take several minutes depending on the lengths of sequences, number of sequences and computer's processing speed. DNA alignment output was exported as (.meg) extension file which was used later to construct the phylogenetic tree. In MEGA5 main menu window, File> open A file/session file was selected as shown in (Labelled number 1) and the exported (.meg) file was opened then from phylogeny menu (labelled number 2) construct/ Test Maximum Neighbour

Joining Tree was selected which opens another dialog window asking to input the analysis preference.

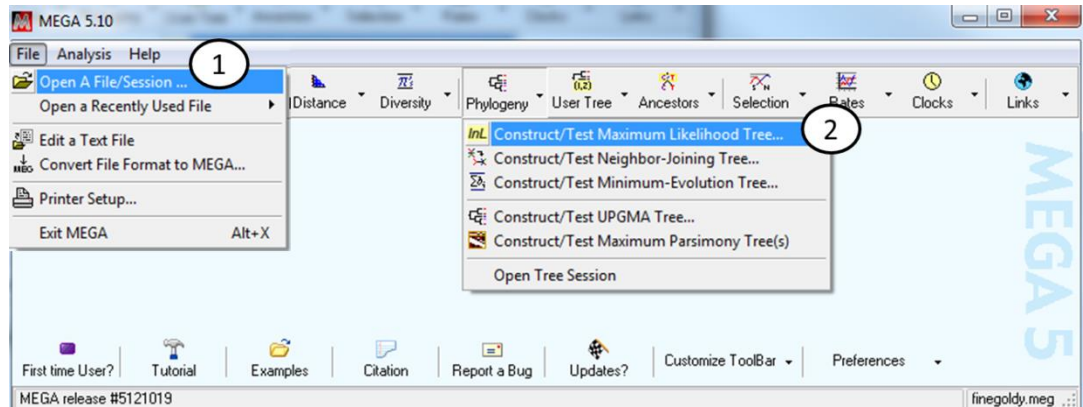


Figure 4.9: A screenshot from MEGA5 main menu showing (1) opening a saved file or session to lead the saved (.meg) file preparing to construct Maximum Likelihood Phylogenetic Tree (2).

Default settings were used and then the Compute button was clicked to start constructing the phylogenetic tree. After finishing the computational process (Figure 4.10), a tree explorer window opened displaying the phylogenetic tree of maximum likelihood which can be displayed a different rectangular, straight curved and circular shapes as well as rooting (see results section for *Bacteroides* phylogenetic tree).

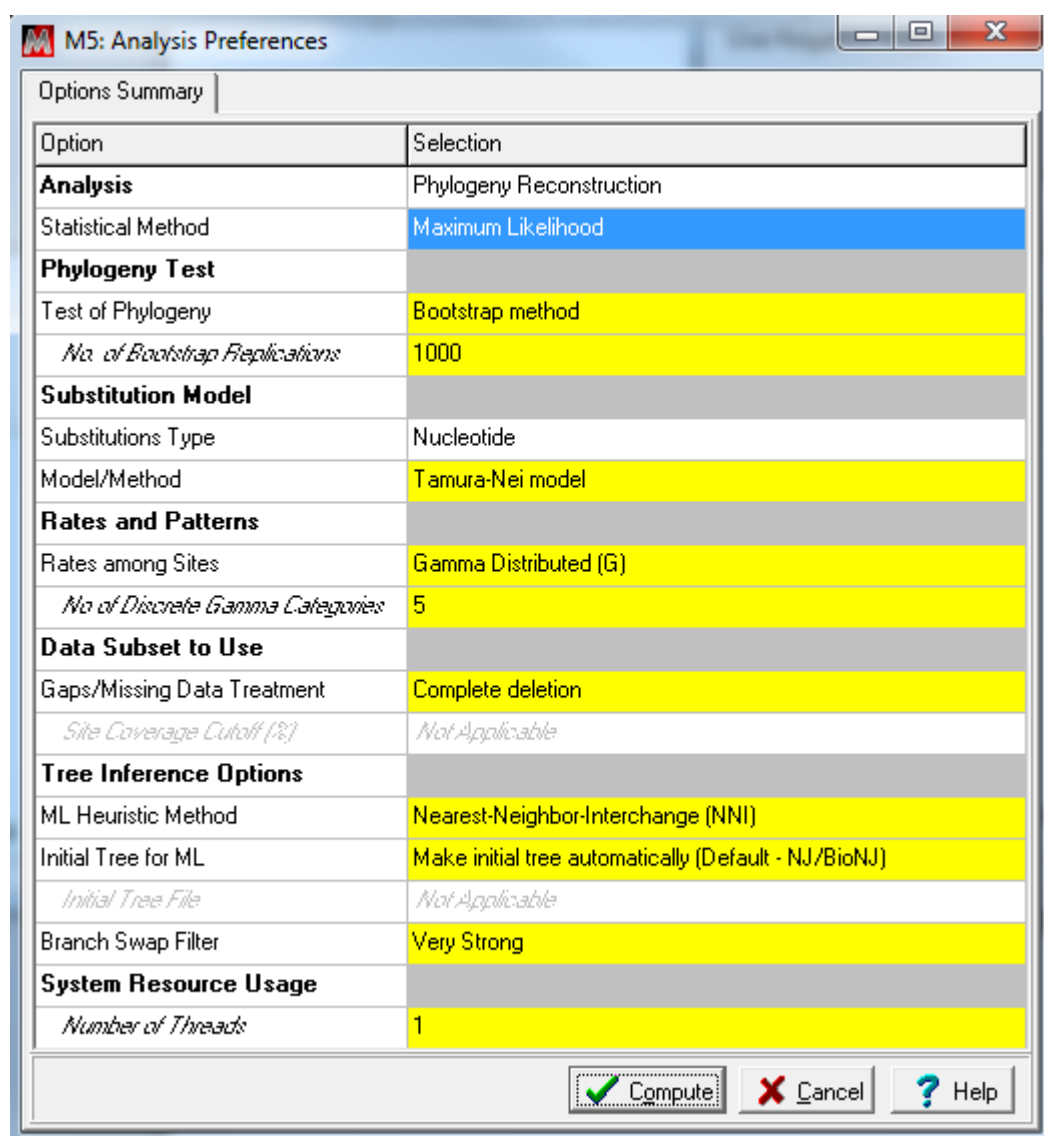


Figure 4.10: A screenshot of default settings of Analysis preference for running Maximum likelihood where 1000 Bootstrap method is selected using Tamura-Nei model.

4.2.10 Evaluation of Microbial Source Tracking (MST)

contamination using *Bacteroides* species as a host specific faecal indicator

Host specific MST method was used to identify whether human faecal contamination source was present in the study area. Highly conserved *Bacteroides* host-specific molecular markers that likely reflect differences in host animal digestive systems have been targeted using PCR-based amplification method by designing primers specific to amplify these markers. These primer sets have been described, and successfully tested in the past two decades showing high specificity and sensitivity to identify and discriminate different faecal contamination sources such as human, cow, sheep, birds and other hosts. (Okabe and Shimazu, 2007, Bernhard and Field, 2000a, Bernhard and Field, 2000b).

4.2.10.1 Study area and water sampling

As results (Chapter 3) show that the stream was the main culprit in contaminating the bathing area, three composite water samples were collected similarly as described in the water sampling section (Chapter 3) from Challaborough stream, two of which were collected randomly and one was collected at a contamination event. Water samples were filtered using the membrane filtration method as described in the materials and methods in chapter three and then membranes were placed on BBE agar plates and incubated for 48 hours in anaerobic cabinet. After colony development,

membranes were transferred into fresh BPRM and incubated for 48 hours to obtain a *Bacteroides* suspension to facilitate DNA extraction.

4.2.10.2 DNA extraction and PCR using *Bacteroides* host specific primers to determine the source of faecal contamination.

Extraction and quality assessment of genomic DNA from *Bacteroides* species suspension was carried out using the method described in section 4.2.9.2. PCR reactions were performed using the method described in PCR section using four primer sets specific to detect general *Bacteroides*, in addition to human, cow and dog host specific *Bacteroides* molecular biomarker.

4.2.10.3 *Bacteroides-Prevotella* universal primer

Bacteroides isolates were confirmed by DNA extraction and performing a PCR technique using Bac 32F and Bac 704R universal *Bacteroides* primer set, the PCR condition was conducted as explained in section 4.4.3.

4.2.10.4 Human specific *Bacteroides* primer set

After confirming the *Bacteroides* isolates using the general Bac 32F and Bac 704R universal *Bacteroides* primer set, another PCR reaction was performed on extracted genomic DNA using human-specific HF183 *Bacteroides* forward primer 5'-ATCATGAGTTCACATGTCCG-3', (Bernhard and Field, 2000b) reverse primer Bac708R 5'-CAATCGGAGTTCTTCGTG-3' (Bernhard and Field, 2000a). Primers were manufactured by Eurofins (MWG BiotechLtd., Germany).

PCR conditions were exactly as described in section 4.4.3 except for annealing temperature was 55.3 °C.

4.2.10.5 Dog specific *Bacteroides* primer set

The presence of dog faeces source was investigated using dog specific *Bacteroides* primer set, kindly provided by Hussein *et al* (in press). Primers were manufactured by Eurofins (MWG BiotechLtd., Germany) and the PCR conditions were exactly as described in 4.4.3 section except for annealing temperature was 62.5.

4.2.10.6 Cow specific *Bacteroides* primer set

PCR amplification of extracted genomic DNA was carried out using specific *Bacteroides* cow CF128F forward primer 5'-CCAACYTTCCCGWTAATC-3' (Bernhard and Field, 2000b) coupled with the reverse primer 5'- Bac708R CAATCGGAGTTCTTCGTG-3' (Bernhard and Field, 2000a). Primer sets were manufactured by Eurofins (MWG BiotechLtd., Germany). PCR conditions were exactly as described in section 4.4.3 except for annealing temperature was 54.8 °C.

4.2.11 *Bacteroides* LPS extraction, profiling and LPS activity in LAL assay and cell culture models

Bacteroides LPS was studied using SDS-PAGE. The information gained from LPS profiling and identification of different *Bacteroides* species present in marine bathing waters will shed light on different *Bacteroides* species LPS

structures present in marine bathing waters and provide valuable information to help understanding and optimising a method to detect the *Bacteroides* species which might be potentially used as a rapid biomarker for recent faecal contamination in marine bathing waters. Different SDS-PAGE methods were tried in this study to profile the LPS of *Bacteroides* species, however only the method mentioned below worked successfully.

4.2.11.1 Lipopolysaccharide extraction

LPS was extracted from an overnight broth culture of *Bacteroides* using a commercially available LPS extraction kit (Chembio, UK). The protocol was followed as instructed by the manufacturer's manual with minor optimisation as the follows:

2 mL of broth culture (2×10^8 CFU mL⁻¹) was transferred to a suitable centrifuge tube, centrifuged for 10 minutes at 13,000 x g at room temperature. The supernatant was discarded and the pellet was washed with LPS-free cell culture grade PBS once and the centrifugation step was repeated once to ensure complete removal of any broth medium remnant. The supernatant was discarded then the pellet was loosened thoroughly by repetitive tapping of the tube before any solution was added, as this step showed its effectiveness in dispersing the pellet with the upcoming reagent additives. One mL of the lysis buffer provided with the LPS extraction kit (Chembio, UK) was added to the pellet then vortexed vigorously until homogenised. After adding 200 µL of chloroform, the suspension was separated into 2 main distinctive layers with a fine white layer due to the differences in density. The tube was centrifuged at

13,000 x g for 10 minutes at 4 °C (Hawk 15/05, MSE, UK) then 400 µL of the supernatant was transferred into a new 1.5 mL microcentrifuge tube without disrupting the lower layer. Afterwards, 800 µL of the purification buffer was added to the tube, mixed then incubated for 10 minutes at -20 °C. The purpose of this step was to purify LPS from other extracts of cells such as protein, nucleic acids and lipids. The suspension then was centrifuged for 15 minutes at 4 °C then the upper layer was discarded. 70% ethanol alcohol was added to wash the LPS pellet to remove any impurities such as salts by inverting the tube 2-3 times then centrifuged for 3 minutes at 13,000 x g at 4 °C. The supernatant was discarded and the pellet left to dry at room temperature then dry weight was measured. LPS pellet was treated with Proteinase K to insure high purity of LPS by adding 2.5 µg of proteinase K per 1 µg of LPS and incubated at 50 °C for 30 minutes. Purification and alcohol washing steps were repeated as described above and LPS pellet was dried, weighted and preserved at -20 °C. Purity of LPS from protein and nucleic acids was investigated by the measurement of UV absorbance at 280 nm for protein and A260/280 and A260/230 ratio to detect nucleic acid using NanoDrop™ spectrophotometer which detected no protein or nucleic acids.

4.2.11.2 LAL assay activity of LPS extracted from *Bacteroides* species

Extracted LPS from five *Bacteroides* species were assessed for activity in the LAL assay. Results in chapter three have shown that *Bacteroides* species increase from virtually zero to the highest recorded number of 2700 CFU 100

mL⁻¹. Results also showed that *Bacteroides* numbers correlated well to the total LPS; hence, it was interesting to investigate the activity of *Bacteroides* LPS and how much it contributes to the activity of total LPS in marine bathing waters. LPS activity was assessed using the LAL assay as described in chapter three. One nanogram of LPS was extracted from five *Bacteroides* species isolated from marine bathing waters in addition to one nanogram of *E. coli* O111:B4 were assayed for their activity using the LAL assay.

4.2.11.3 Sodium Dodecyl Sulphate Polyacrylamide Gel

Electrophoresis (SDS-PAGE)

A modified method of discontinuous Laemmli's (1970) SDS-Page method where 10% acrylamide gel and tris-glycine buffer with low SDS (Table 4.2) was used to separate the LPS of *Bacteroides* species isolated from marine bathing waters. All chemicals were purchased from Sigma Aldrich unless stated otherwise.

Table 4.2: Modified SDS-PAGE method based on Laemmli's recipe

Running buffer	
Substance	Weight
Glycine	47.5 g
Tris	7.5 g
SDS	0.1 g
Q.S to 500 mL deionised water	
10% acrylamide	
Substance	Volume
Acrylamide/Bis-acrylamide 29:1	3.33 mL
5 x Running buffer (Stock)	2 mL
Distilled water	4.56
Ammonium persulfate (APS)	100 µL
Temed	6 µL
Sample Buffer x6	
Substance	Volume
Glycerol	3 mL
Bromophenol blue x10 (Tracking dye)	100 µL
Running Buffer x1	6.9 mL

4.2.11.4 *Bacteroides* LPS gel electrophoresis

Bacteroides LPS gel electrophoresis was performed using ATTO Vertical electrophoresis systems model AE-6200 Slab EP Chamber (Japan) and assembled according the manufacturer's manual. LPS samples were prepared by adding 1 mL of sample buffer to 1 mg of LPS then heated in a heat block for 30 minutes until LPS pellet completely dissolved. Samples were mixed then 20 µL run on 10% polyacrylamide gels. Gel solutions were poured carefully using a

5 mL pipette to avoid air bubbles and left to solidify. All gels were run at 120 volts for one and a half hours in 300 mL for upper chamber, 350 mL for lower chamber running buffer. Several runs of LPS electrophoresis were carried out on most *Bacteroides* isolates; a sample is shown in the results section (Figure 4.18). Similarly, LPS extracted from *E. coli* isolated from seawater and run on SDS-PAGE for comparison. Staining of polyacrylamide gels was achieved by placing each gel in appropriate sized staining tray containing silver stain solution (Bio-Rad Laboratories, Italy) as instructed by the manufacturer's instructions. Gels were visualised using *Bio-Rad* UV transilluminator CHEMI DOC XRS gel imaging system (Bio-Rad Laboratories, Italy).

4.2.11.5 Statistical analysis of LPS profiling and similarity using Image J and Primer 6 software

LPS profiling and similarity of *Bacteroides* species was extensively studied in this chapter in terms of LPS banding pattern to determine whether different *Bacteroides* species present in the seawater possess similar LPS which can be targeted as a potential faecal-specific biomarker. Analysis of LPS gel electrophoresis was conducted using ImageJ and statistical analysis software Primer 6 (Waines *et al.*, 2011, Moreno *et al.*, 2006). LPS gel electrophoresis images were exported as .TIF image files and were viewed in ImageJ, an online software that widely used in Image analysis as shown in figure 4.11

(<http://rsb.info.nih.gov/ij/download/win32/ij146-jdk6-setup.exe>).

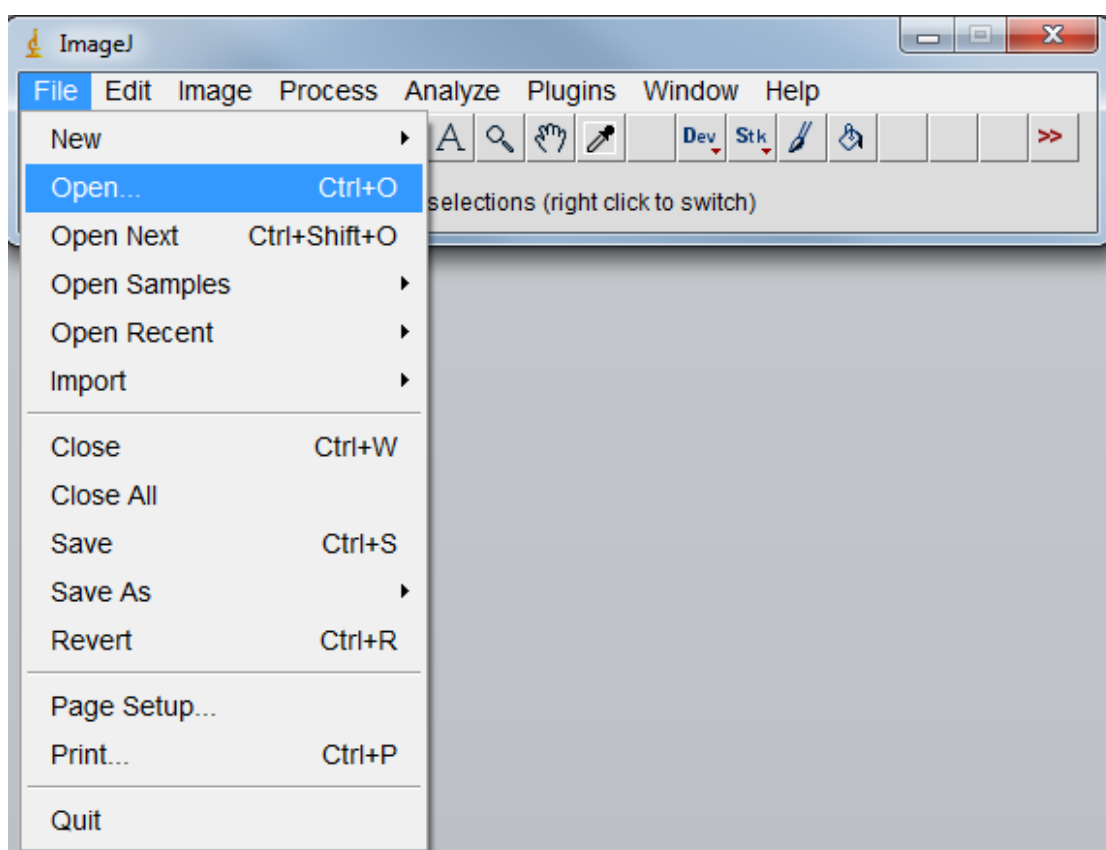


Figure 4.11: A screenshot from ImageJ homepage showing importing images for further analysis.

LPS electrophoresis images were manipulated using the crop function before printing. Bands were given numerical sequences depending on their migration distance and depending on *E. coli* LPS banding pattern as it has been used as a marker for absence and presence of bands. Lanes were then selected using rectangular selection tool, firstly, the first lane was selected and was given a numerical number 1, secondly, the second lane was selected and was given number 2, thirdly, all lanes were selected similarly to the second step except for the last lane which was given number 3, by pressing number three and arbitrary graphical representation of band intensity presented in the form of peaks and each peak represents a single band. Peaks were printed and compared to the

printed numbered bands for accurate labelling of these peaks. The area of each peak value (number of pixels) was estimated using the Wand (tracing) tool generating area numbers which then exported to Microsoft Excel 2010. Statistical analysis of the area values were conducted using Primer 6 (Plymouth University, UK). Statistical analysis conducted were resemblance analysis using S17 Bray Curtis similarity, Non-metric Multi-Dimensional Scaling (MDS) was used to represent relative similarities between different *Bacteroides* species LPS Band patterns and compared to *E. coli* LPS banding pattern. Cluster analysis was conducted to visualise group similarities and (ANOSIM) analysis was conducted for similarity of gel replicates. Primer 6 (version 6.1.10) software was used to show abundance and similarities between gel electrophoresis bands, data obtained from image J was exported to primer 6 and was analysed. From the main menu select analyse then resemblance analysis Resemblance measure: S17 Bray Curtis similarity and half a matrix appears showing resemblance percentage between each LPS profiling.

4.2.11.6 Immunological activity of *Bacteroides* LPS in cell culture models

The aim of this study was to have an insight of the severity of immune response which is reflected by stimulating MM6 and MPI cells with five extracted *Bacteroides* species LPS and measuring the release of proinflammatory cytokines. MM6 and MPI cells were used in this study as a model to investigate *in vitro* immune responses induced by the extracted LPS of those *Bacteroides* species. A positive control represented by a 10 ng mL⁻¹ of commercially

available *E. coli* K12 LPS was used. Negative controls were also run represented by cells only without any treatments.

4.2.11.6.1 MM6 cell culture experimental setup

General maintenance of MM6 cell culture is described in chapter 2.

A final concentration of 0.5×10^6 cells per mL was transferred to each well of a 24 well plate (Nunc, UK). Two plates were prepared to assess the release of two proinflammatory cytokines; TNF- α and IL-6. Cells were stimulated with a final concentration of 1000, 100 and 10 ng mL⁻¹ extracted LPS from five identified *Bacteroides* species by adding 10 μ L of each serially diluted sample in duplicate and running three independent experiments. plates were incubated for 6 hours for TNF- α release and for 24 hours for IL-6 production (Jackson *et al.*, 2008). Supernatant was collected after the proper incubation period by aspirating well content into 1.5 mL sterilised microcentrifuge tubes then spun at 160 x g for 5 minutes. Supernatant was then transferred into 2 microcentrifuge tubes and kept in -20 °C until assaying using sandwich ELISA.

4.2.11.6.2 IL-6 and TNF- α sandwich ELISA for the human MM6 cell line

ELISA assays to detect IL-6 and TNF- α cytokines were optimised using a checkerboard titration method using the concentration range recommended by the manufacturer. ELISA 96-well microtiter plates (MaxiSorp®, Thermo Scientific, UK) were manually coated with the capture antibody for the target cytokines (Rat anti-human IL-6 Mab IgG1 or Rat anti-human TNF- α Mab1 IgG1

commercially available from BD-Pharmingen Oxford, UK) by adding 50 μL per well of 1 $\mu\text{g mL}^{-1}$ in Phosphate Buffered Saline (PBS pH 7.3, Oxoid, UK) and left overnight at 4 $^{\circ}\text{C}$. Microtiter plates were washed three times with PBS containing 0.05% Tween[®] 20 / PBS washing buffer (Tween[®] 20, Sigma Aldrich, UK) prepared with double distilled water then 150 μL per well of 2% Bovine Serum Albumin (BSA-PBS) blocking buffer was added to block any non-specific adsorption sites for other proteins followed by incubating the plates 4 hours at room temperature. Plates were then washed three times with the washing buffer. A set of standards ranging 6-5000 pg mL^{-1} as well as appropriate dilutions of cell culture medium of stimulated MM6 cells samples were prepared and 50 μL per well was added in duplicate as recommended by the manufacturer. Microtiter plates were incubated overnight at 4 $^{\circ}\text{C}$. Detection antibody (BD-Pharmingen Oxford, UK) was diluted to 0.5 $\mu\text{g mL}^{-1}$ in 2% BSA-PBS, then 50 μL per well was added after a 3 times washing step and then plates were incubated for 4 hours at room temperature. After 4 hours, plates were washed three times with washing buffer. Next, 50 μL per well of Streptavidin- horseradish peroxidase (HRP, R&D systems, UK) at 1/250 dilution in 2% BSA- PBS was added and left for 1 hour at room temperature. After one hour incubation, microtiter plates were washed three times with the washing buffer and a 100 μL per well of 3,3',5,5'-Tetramethylbenzidine (TMB, 0.4 g L^{-1}) substrate reagent was added (TMB Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). After colour development for 15 minutes at room temperature, the reaction was stopped with 50 μL per well of 1N H_2SO_4 . Microtiterplates were read for absorbance on 450 nm using a microplate reader (VersaMax[™], Molecular Devices, Sunnyvale, CA) and SoftMax pro (software version 5.4).

4.2.11.6.3 MPI cell culture experimental setup

MPI cells are newly described self-renewing, GM-CSF dependent non-transformable lung alveoli macrophage-like cells (Fejer *et al.*, 2013). MPI cells were used in this study as a model to investigate *in vitro* immune response induced by *Bacteroides* species LPS, since MPI are alveoli macrophage-like cells, it could shed light on the potential immunological response represented by the production of proinflammatory cytokine as macrophages are multifaceted cell types in the first line of antimicrobial defence. 180 μL were seeded in each well of a 96 well plate giving a final concentration of 1×10^5 cells per well. Cells were stimulated in duplicate with 20 μL of a final concentration of 1000, 100 and 10 ng mL^{-1} extracted LPS from five identified *Bacteroides* species. Plates were incubated for 6-8 hours for TNF- α release and for 24 hours for IL-6 production.

4.2.11.6.4 IL-6, TNF- α and IL-1 α sandwich ELISA for MPI mouse macrophage cells

ELISA 96-well microtiter plates (MaxiSorp[®], Thermo Scientific, UK) were coated manually with the capture antibody (anti-rat IL-6 Mab IgG1, anti-rat TNF- α Mab IgG1 or anti-IL-1 α Mab IgG1, eBioscience) by adding 50 μL per well of 4 $\mu\text{g mL}^{-1}$ for the TNF- α and 4 $\mu\text{g mL}^{-1}$ for IL-6 and IL-1 α in Phosphate Buffered Saline (PBS pH 7.3, Oxoid, UK) and left overnight at 4 $^{\circ}\text{C}$. Microtiter plates were washed three times with 0.05% Tween[®] 20 / PBS washing buffer (Tween[®] 20, Sigma Aldrich, UK) prepared with double distilled water then a 150 μL per well of 2% Bovine Serum Albumin (BSA-PBS) blocking buffer was added, then the plates were incubated for 1 hour at room temperature. Plates were then washed

3 times with the washing buffer. A set of standards ranges from 15-2000 pg mL⁻¹ of recombinant TNF- α for the TNF- α assay and 15-1000 pg mL⁻¹ of recombinant IL-6 and IL-1 α as well as appropriate dilutions of samples were prepared and 50 μ L per well was added in duplicate. Microtiter plates were incubated overnight at 4 °C. Biotinylated TNF- α , IL-6 or IL-1 α detection antibody was diluted to 2 μ g mL⁻¹ in PBS, then 50 μ L per well was added to the plates, plates were then incubated for 1 hour at room temperature. After 1 hour, plates were washed 3 times with washing buffer. Next, 50 μ L per well of Avidin-HRP diluted 1:500 (eBioscience, UK) at 1/500 dilution in 2% BSA- PBS and left for 1 hour at room temperature. After one hour incubation, microtiter plates were washed 3 times with the washing buffer and a 50 μ L per well of super aqua blue (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, eBioscience, UK) ELISA substrate was added. After colour development at room temperature microtiterplates were read for absorbance on 405 nm using the same plate reader and software as detailed in section 2.8.1.1. Results were calculated from the standard curve run simultaneously with each run. Standard curve was constructed using four parametric logistic curve. ELISA materials were kindly provided from Dr. Gyorgy Fejer (Centre for Biomedical Research - Translational & Stratified Medicine Peninsula Schools of Medicine and Dentistry, Plymouth University).

4.2.11.7 ELISA assay to detect *Bacteroides* lipopolysaccharides as a faecal-specific biomarker

An assay was designed to detect *Bacteroides* LPS using anti- *Bacteroides* LPS antibody was attempted. The assay was ELISA based and both Sandwich and direct ELISA technique were attempted.

4.2.11.7.1 Sandwich ELISA assay to detect *Bacteroides* LPS

Sandwich ELISA was attempted to detect purified LPS of *Bacteroides fragilis* type culture NCTC 9343 using *Bacteroides fragilis* LPS monoclonal antibody (Novus Biological, UK). Antibody was aliquoted immediately when received and stored at -20 °C according to the manufacturer's instructions. A checkerboard ELISA method was used to optimise the concentration of capture and detection antibodies. Plates were coated with detection Ab (non Biotinylated) at a constant concentration of 1 µg mL⁻¹ overnight at 4 °C. Plates were washed three times with PBS containing 0.05% v/v Tween[®] 20 (washing buffer) and blocked with 1% BSA-PBS by adding 300 µL per well using a multi-channel pipet and Incubate for 2 hours at room temperature. After a washing step, a standard curve was prepared by preparing one fold dilution starting from 1 µg mL⁻¹, and 100 µL per well was added and incubated overnight at 4 °C. Plates were washed three times with the washing buffer then the detection antibody (NB 100-64513B Biotinylated) was added at concentrations 1 µg mL⁻¹, 0.5 µg mL⁻¹, 0.25 µg mL⁻¹ and 0.125 µg mL⁻¹ diluted in PBS-Tween[®] 20 1% BSA and left for 2 hours at room temperature. Plates were washed three times with the washing buffer followed by adding 50 µL per well of HRP-conjugated

streptavidin at 1/2000 dilution in PBS/1% BSA then incubate for one hour at room temperature. Plates were washed three times with the washing buffer and then 100 μ L of detection substrate TMB was added for 30 minutes away from light. The reaction was stopped with 50 μ L of H₂SO₄. Microtiterplates were read for absorbance on 450nm using microplate reader (VersaMax™, Molecular Devices, Sunnyvale, CA) and SoftMax pro (software version 5.4).

4.2.11.7.2 Direct ELISA assay to detect *Bacteroides* LPS

Sandwich ELISA was attempted to detect *Bacteroides fragilis* type culture NCTC 9343 purified LPS/or whole cells using *Bacteroides fragilis* LPS monoclonal antibody (Novus Biological, UK).

A different approach in detecting the LPS of *Bacteroides fragilis* was attempted using direct ELISA assay. Based on a previous study conducted by Péterfi and Kocsis (2000) which describes different coating materials and techniques and explore the best substances and reagent for performing ELISA detection of rough form LPS. In this study, the best pre-coating and blocking agent which is suitable with typical rough LPS (*Bacteroides fragilis* NCTC 9343 LPS) was selected depending on Péterfi and Kocsis (2000) findings and applied in performing the direct ELISA assay. ELISA plates were pre-coated with a 100 μ L of weight Poly-L-Lysine (MW. 260 000, 10 μ g mL⁻¹) in PBS (0.01 M at pH 7.2) overnight at room temperature. Poly-L-Lysine is a polymer molecule that is useful to prepare surfaces for cell and in this case; LPS immobilisation. The mechanism of Poly-L-Lysine is based on the ionic interaction between the polyanionic surfaces and the polycationic layer of Poly-L-Lysine. After washing

the excess of unbound Poly-L-Lysine, a checkerboard ELISA method was conducted to optimise the assay. A standard curve was prepared by preparing one fold dilutions starting from $1 \mu\text{g mL}^{-1}$, and 100 μL per well was added and incubated overnight at 4°C . Plates were washed three times with the washing buffer then the detection antibody (NB 100-64513B Biotinylated) was added at concentrations $1 \mu\text{g mL}^{-1}$, $0.5 \mu\text{g mL}^{-1}$, $0.25 \mu\text{g mL}^{-1}$ and $0.125 \mu\text{g mL}^{-1}$ diluted in PBS-Tween[®] 20 1% BSA and left for 2 hours at room temperature. Plates were washed three times with the washing buffer followed by adding 50 μL per well of HRP-conjugated streptavidin at 1/2000 dilution in PBS/1% BSA then incubate for one hour at room temperature. Plates were washed three times with the washing buffer and then 100 μL of detection substrate TMB was added for 30 minutes away from light. Reaction was stopped with 50 μL of H_2SO_4 . Microtiterplates were read for absorbance at 450 nm using microplate reader (VersaMax[™], Molecular Devices, Sunnyvale, CA) and SoftMax pro software version 5.4.

4.2.11.7.3 Troubleshooting of Anti *Bacteroides* LPS antibody efficiency.

Dot blot technique was performed to assess the reactivity of unbound and biotinylated anti *Bacteroides* LPS antibodies against purified LPS of *Bacteroides fragilis* NCTC 9343.

Different formats of dot blot were used:

Ten microliters of purified *Bacteroides fragilis* LPS (1000, 100 and 10 ng mL⁻¹) applied directly on a membrane as a dot defined by a pencil on two 0.2 µm PVDF membranes (Millipore, UK) and were left to completely dry at room temperature. Membranes were blocked for any non-specific sites by soaking in a petri dish containing 5% BSA in tris-buffered saline and Tween[®] 20 for 1 hour at room temperature (TBS-T as described in chapter 2). The membranes were transferred into a new petri dish containing 1 µg mL⁻¹ of biotinylated anti *Bacteroides fragilis* LPS Antibody (Novus Biological, UK) on a shaker for 1 hour at room temperature, the other membrane was incubated at 4 °C on a roller (50 mL Falcon[™] tube). Membranes were then washed 3 times for 10 minutes each and incubated with Streptavidin-HRP for 1 hour at room temperature. Membranes were washed 3 times for 10 minutes each and a Bio Rad developing substrate was added. No result was obtained. Similarly, five extracted and purified LPS from *Bacteroides* species were applied and the same procedure was followed, resulting in no reaction. This step was conducted to assess whether different *Bacteroides* species react with the anti-*Bacteroides* LPS antibody.

It has been thought that maybe the biotinylation process (antibody was biotinylated by the manufacturer, Novus Biologicals, UK) might interfere with the reactivity of the antibody, hence, a slightly modified experiment to the above mentioned was conducted. Briefly, extracted LPS from five different *Bacteroides* species were placed on 2 PVDF membranes (Millipore, UK) and left to completely dry at room temperature. Membranes were blocked with 5% BSA TBS-T. The membranes were incubated with *Bacteroides fragilis* LPS Antibody

(un-Biotinylated) on a shaker for 1 hour at room temperature and the other membrane at 4 °C on a roller. Membranes washed three times for 10 minutes each and incubated with HRP conjugated Anti mouse IgG antibody for 1 hour at room temperature. Membranes were washed three times for 10 minutes each and a bio Rad developing substrate was added. Unfortunately, No result was obtained.

To assess whether the streptavidin-hrp bridge was working with the biotinylated *Bacteroides fragilis* LPS antibody, this biotinylated antibody was directly added to a PVDF membrane and left to completely dry. The membrane was then blocked with 5% BSA TBS-T for 1 hour and then streptavidin-hrp was added and incubated with the membrane for 1 hour at room temperature. The membrane was washed three times for 10 minutes each and then the developing substrate was added. Results show clear and crisp dots which shows the bridging is taking place.

A different approach was applied by incubating extracted LPS with the anti-*Bacteroides* LPS antibody for 2 hour at room temperature and then the LPS activity was assayed using the LAL assay.

4.3 Results

4.3.1 Phenotypic and genotypic methods for the characterisation of *Bacteroides* species isolated from bathing seawater

A hundred and one *Bacteroides* pure culture isolated from marine bathing water were initially speciated using conventional phenotypic and genotypic methods (using the methods described earlier in the materials and methods section). All *Bacteroides* isolates showed negative reaction when stained with Gram stain. *Bacteroides* species were grouped into five *Bacteroides* groups depending on their indole test reaction followed by their ability to utilise and grow in RPMI broth culture containing rhamnose, trehalose and salicin sugars (Bergey and Holt, 1994). All 101 isolates were confirmed as *Bacteroides* species using 32F and 704R universal *Bacteroides* primer set, a sample of the *Bacteroides* (which were subsequently sequenced) in figure 4.12.

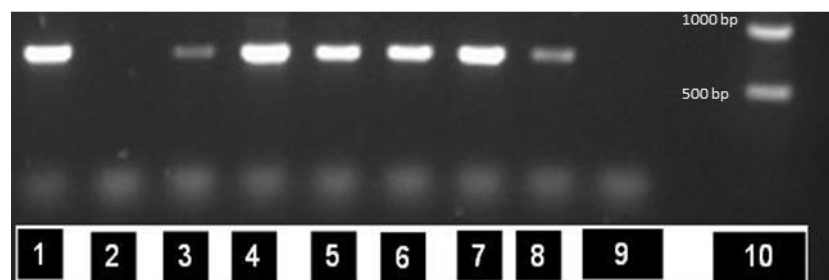


Figure 4.12: Agarose gel electrophoresis showing PCR amplicons using general *Bacteroides-Prevotella* primer set Bac 32F and Bac 708R, 1- *B. fragilis* positive control, 2- Negative control, 3- *B. caccae*, 4- *B. ovatus*, 5- *B. xylanisolvens* 6- *B. fragilis*, 7- *B. fragilis* NCTC 9434, 8- *B. finegoldii* , 9- Negative control, 10- Molecular weight marker.

Randomly selected isolates from each group were further identified by amplifying the 16s rRNA using 27f and 1492r bacteria universal primers to confirm the speciation. Sequencing results showed five distinct *Bacteroides* species, phylogenetic tree was constructed using MEGA5 software (as described in materials and method) is shown in figure 4.13.

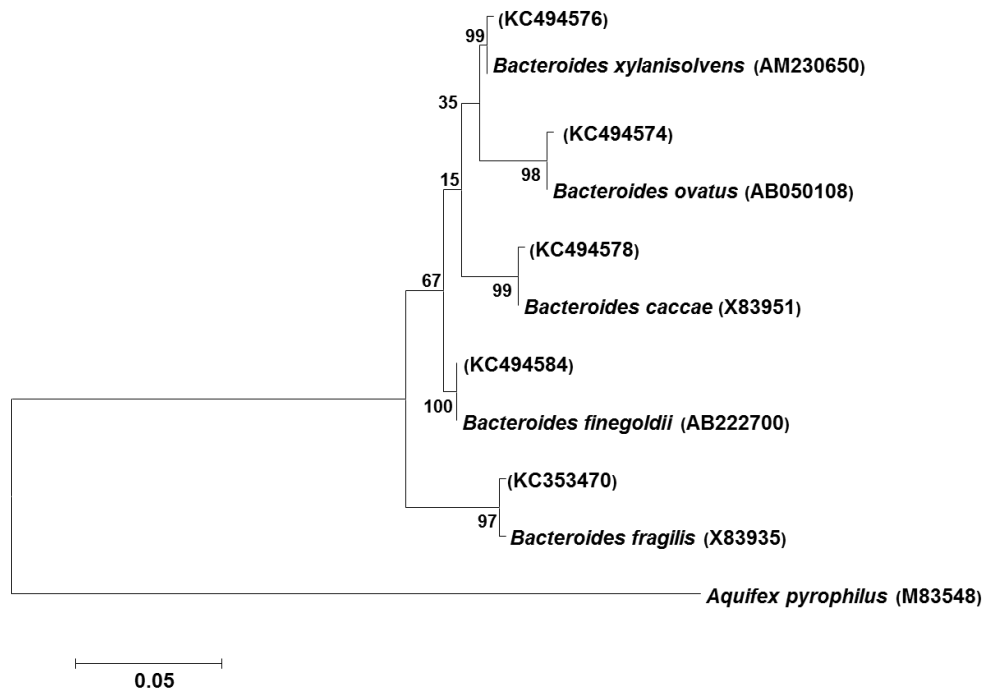


Figure 4.13: Maximum-likelihood tree showing the phylogenetic positions of *Bacteroides* isolates from marine bathing waters based on 800 nt of the 16S rRNA gene sequences, using the Tamura–Nei model. Bootstrap values (%) are given from 1000 replicates. Bar, 5 difference per hundred. GenBank accession numbers are given in parentheses.

4.3.2 Evaluation of microbial source tracking (MST) contamination using *Bacteroides* species as a host specific faecal indicator

Since it has been observed that the main source of contamination was from the stream, three water samples randomly collected from the stream were investigated for the presence and the source of *Bacteroides* host using initially Bac 32F and 704R to confirm as *Bacteroides* followed by *Bacteroides* host specific to cow, dog and human. PCR results showed a positive result

confirming the presence of *Bacteroides* species with the generic Bac32f and Bac708R primers sets (Figure 4.14)

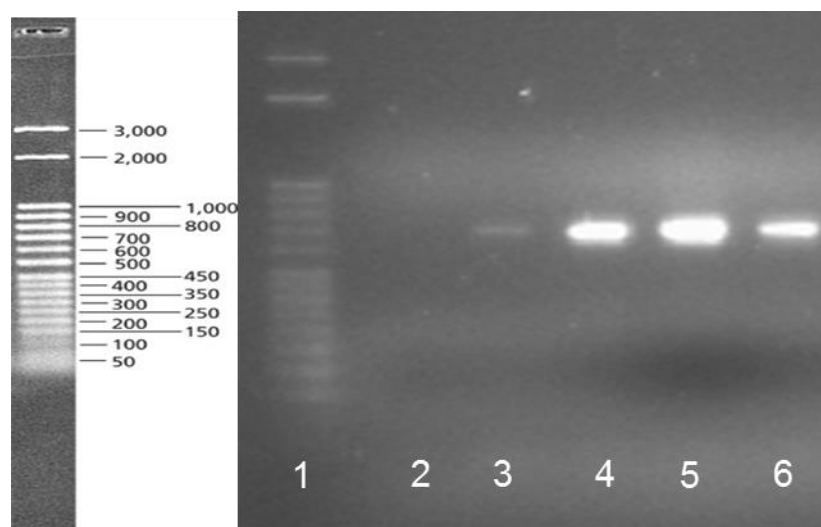


Figure 4.14: Agarose gel electrophoresis showing PCR amplicons using *Bacteroides* 32F general primer 670 bp 1= 50 bp DNA ladder, 2= Negative control, 3= Positive control, 4= stream 1, 5= stream 3, 6= stream 2.

PCR results also showed the presence of human, cow and dog host specific *Bacteroides* biomarkers in all three samples (Figure 4.15, 4.16 and 4.17).

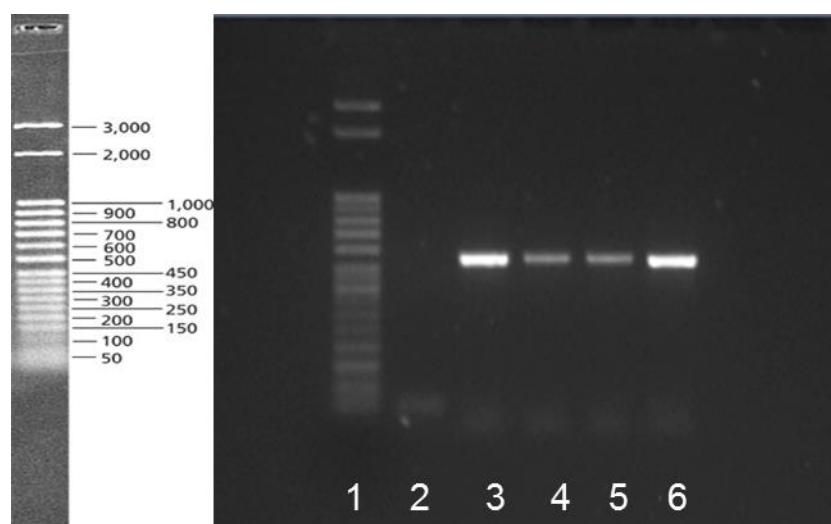


Figure 4.15: Agarose gel electrophoresis showing PCR amplicons using Human-specific *Bacteroides* primer 520 bp. 1= 50 bp DNA ladder, 2= Negative control, 3= Positive control, 4= stream 1, 5= stream 2, 6= stream 3.

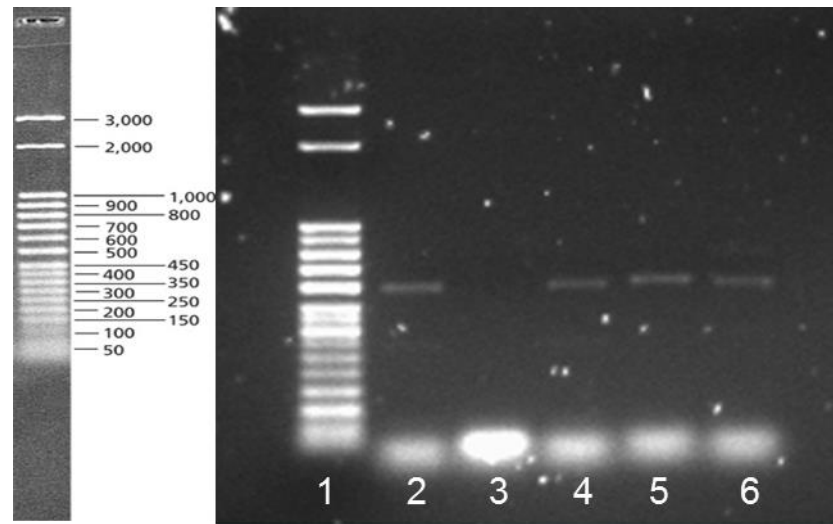


Figure 4.16: agarose gel electrophoresis showing PCR amplicons using Cow-specific *Bacteroides* primer 580 bp. 1= 50 bp DNA ladder, 2= Positive control, 3= Negative control, 4= stream 1, 5= stream 2, 6= stream 3.

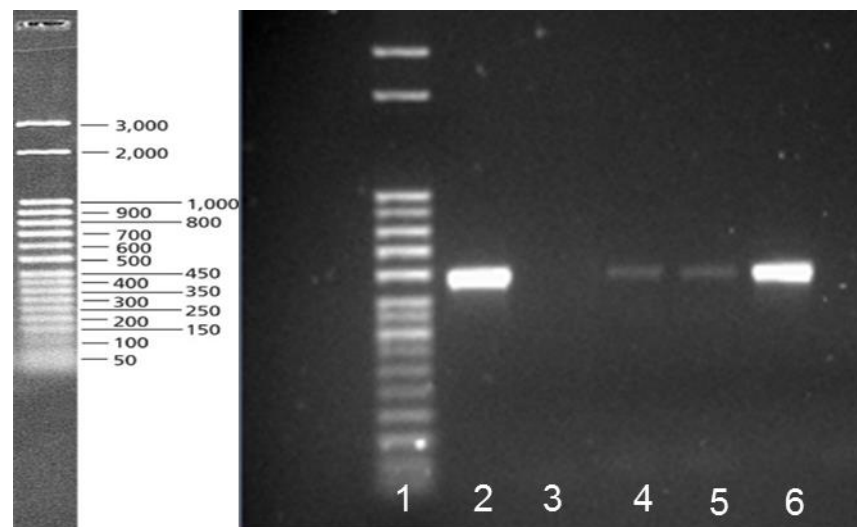


Figure 4.17: Agarose gel electrophoresis showing PCR amplicons using Dog-specific *Bacteroides* primer 572 bp 1= 50 bp DNA ladder, 2= Positive control, 3= Negative control, 4= stream 1, 5= stream 2, 6= stream 3.

4.3.3 LAL activity of LPS extracted from *Bacteroides* species

Extracted LPS from five *Bacteroides* species was assessed for activity in the LAL assay. Results show that the activity of LPS among *Bacteroides* species isolates was highly similar and extremely low potency in comparison to *E. coli* LPS reference as shown in table 4.3.

Table 4.3: LAL activity of five extracted LPS from *Bacteroides* LPS expressed in EU mL⁻¹. *E. coli* LPS was used as reference for comparison. N=3, \pm represent standard deviation of the mean.

Extracted LPS (1 ng)	LAL activity EU ng ⁻¹
<i>Bacteroides fragilis</i>	0.030 \pm 0.001
<i>Bacteroides fingoldii</i>	0.029 \pm 0.000
<i>Bacteroides caccae</i>	0.030 \pm 0.000
<i>Bacteroides xylanisolvens</i>	0.029 \pm 0.001
<i>Bacteroides ovatus</i>	0.025 \pm 0.007
<i>E. coli</i> O111:B4	10 \pm 0.2

4.3.4 LPS profiling using SDS-PAGE

Lipopolysaccharides from *Bacteroides* species isolated from marine bathing water were extracted (using the method described in the materials and methods section) and profiled for their LPS pattern similarity was performed using SDS-PAGE method. LPS from three *E. coli* strains U 5/41 strains also isolated from the same water samples was extracted and run for LPS banding pattern similarity as a reference for comparison. Results of LPS electrophoresis is shown in figure 4.18.

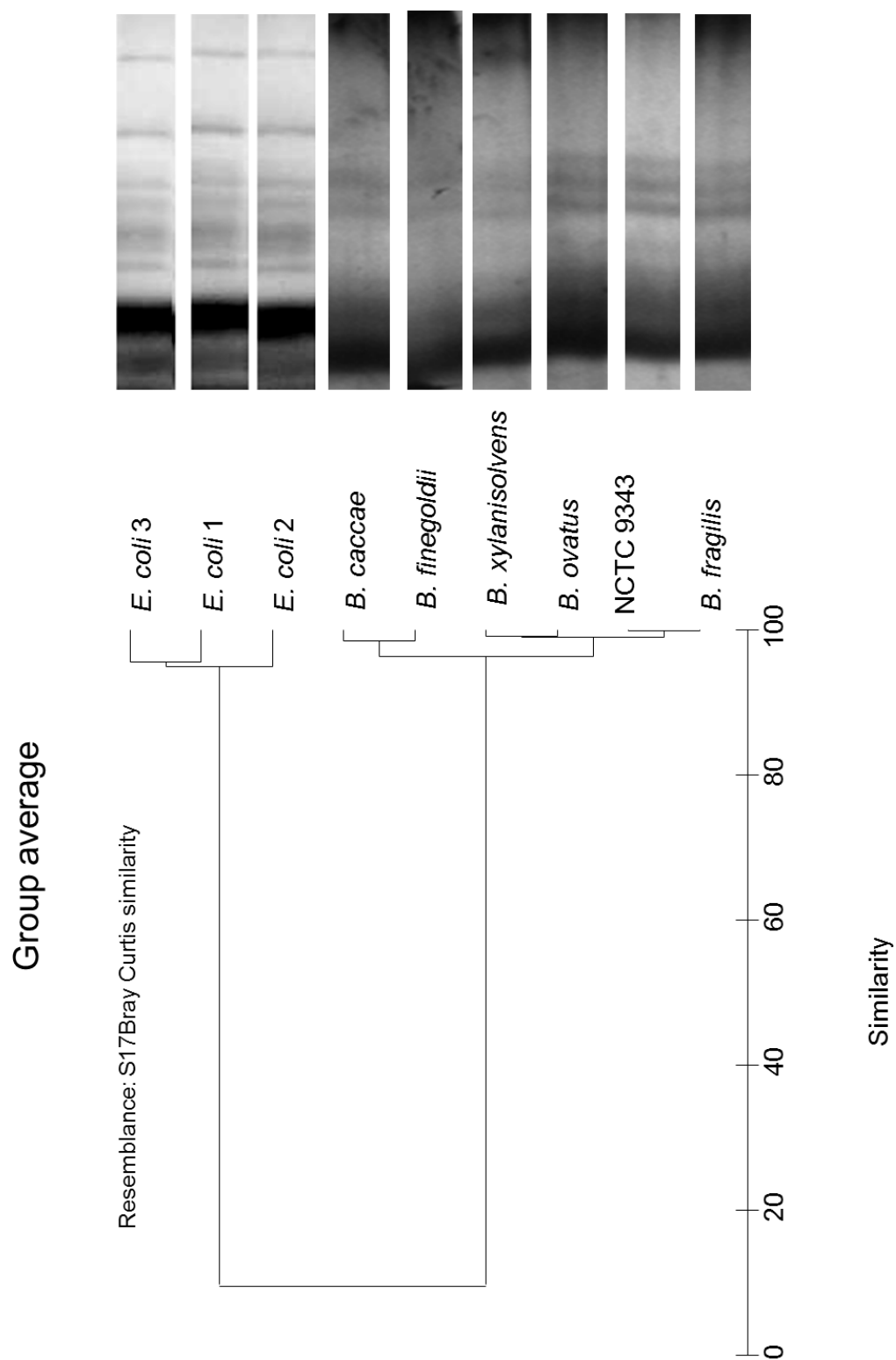


Figure 4.18: LPS obtained by extraction from *Bacteroides* species/ or *E. coli* strains isolated from marine bathing waters and subjected to SDS-PAGE and stained by silver stain method. Similarity percentage using Bray Curtis similarity analysis in LPS pattern is shown underneath each gel lane.

Results showed that there was a high similarity in the LPS banding pattern across the *Bacteroides* species (<95% similarity) and a very low similarity when compared to the LPS pattern of *E. coli* (less than 10%). Result also confirmed that profiled *Bacteroides* species have a similar rough type of LPS. Figure 4.19 also showing the similarity in a half matrix graph.

Similarity (0 to 100)

	E. coli 1	E. coli 2	E. coli 3	B. ovatus	B. xylanisolv	B. fragilis	NCTC 9343	B. fingoldii	B. caccae
E. coli 1									
E. coli 2	95.34								
E. coli 3	95.544	94.447							
B. ovatus	9.3002	8.4481	10.312						
B. xylanisolv	9.1753	8.3137	10.186	99.068					
B. fragilis	9.7997	8.9635	10.839	98.723	98.958				
NCTC 9343	9.7589	8.9208	10.797	98.9	99.033	99.795			
B. fingoldii	10.053	9.2134	11.122	96.596	97.09	97.149	97.147		
B. caccae	10.371	9.5366	11.466	95.087	95.708	95.895	95.891	98.457	

Figure 4.19: Half a matrix showing similarity in LPS banding pattern in SDS-PAGE between five *Bacteroides* species and three *E. coli* isolated from marine bathing waters.

4.3.5 Immunological activity of *Bacteroides* LPS in cell culture models

Lipopolysaccharides from five *Bacteroides* species were extracted and used as stimulants in two cell culture models in three independent experiments to assess their biological activity by monitoring the levels of proinflammatory cytokines.

4.3.5.1 Immunological activity of *Bacteroides* LPS in MPI cells

Results showed a dose response effect of LPS in the production of IL-1 α in the MPI cells. In addition, it seems that the potency of *Bacteroides* LPS is more than 100 fold less potent in comparison to the *E. coli* K12 LPS (Figure 4.20).

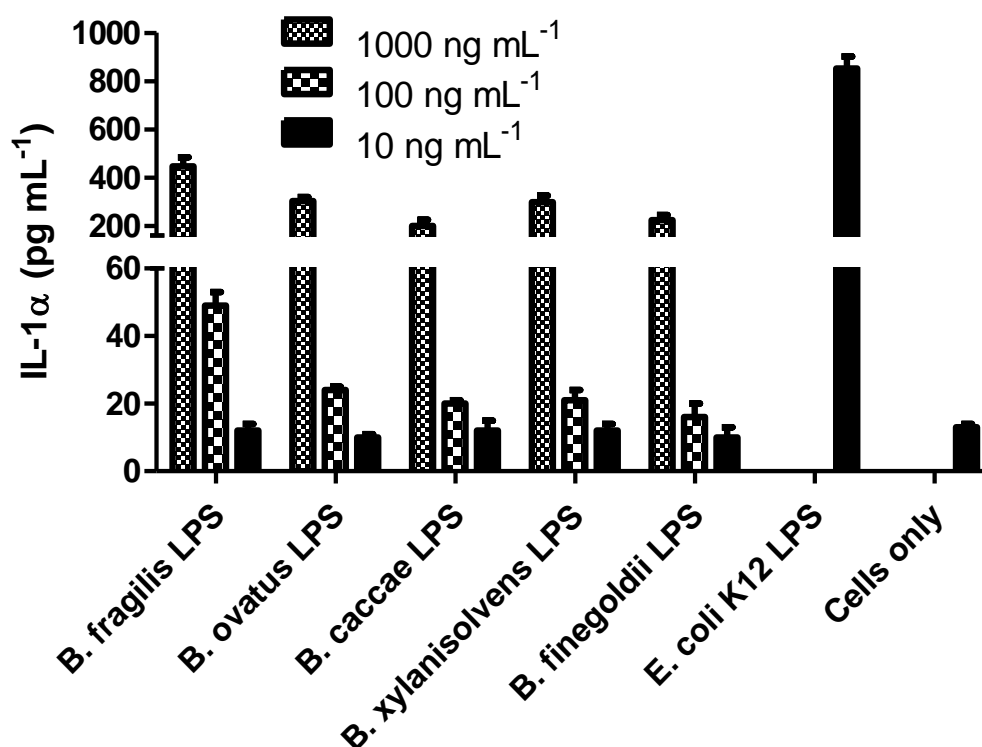


Figure 4.20: Dose response of lipopolysaccharide (LPS) induced IL-1 α production from stimulated MPI cells measured using sandwich ELISA. Cells were stimulated with 10, 100, and 1000 ng mL⁻¹ of five *Bacteroides* species extracted LPS and 10 ng mL⁻¹ of *E. coli* K12 LPS was used as a positive control. Bars represent the mean and standard error of 3 independent experiments.

Results showed a dose response effect of LPS in the production of TNF- α . Moreover, it appears that the potency of *Bacteroides* LPS is greater than 100 fold less potent in comparison to the *E. coli* K12 LPS (Figure 4.21).

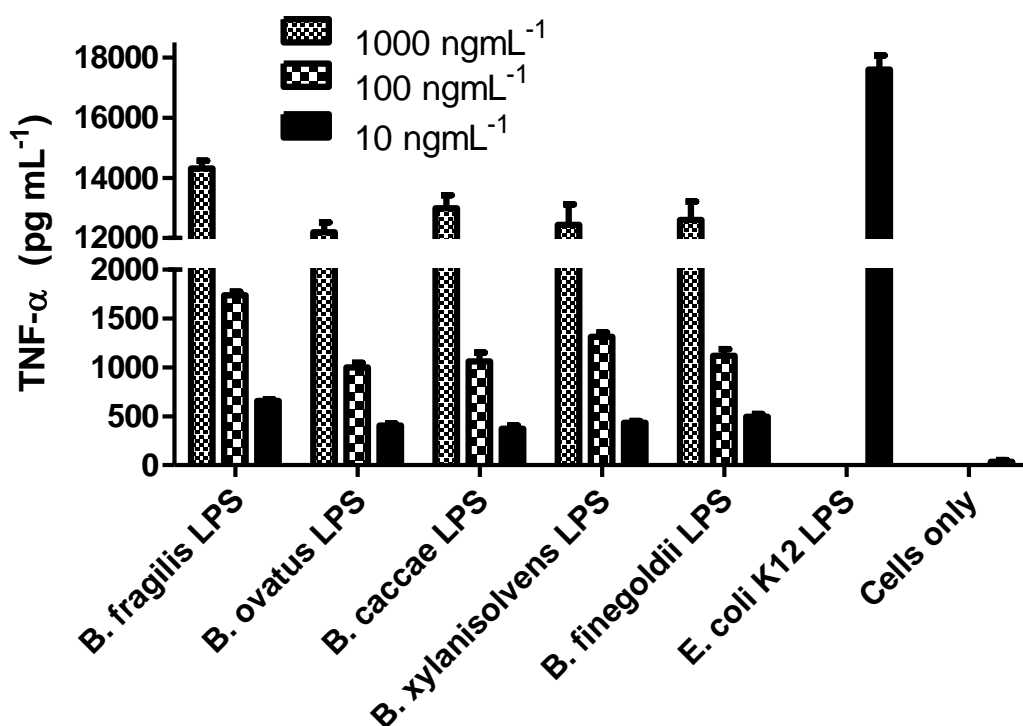


Figure 4.21: Dose response of lipopolysaccharide (LPS) induced TNF- α production from stimulated MPI cells measured using sandwich ELISA. Cells were stimulated with 10, 100, and 1000 ng mL⁻¹ of five *Bacteroides* species extracted LPS and 10 ng mL⁻¹ of *E. coli* K12 LPS was used as a positive control. Bars represent the mean and standard error of 3 independent experiments.

Results also showed a dose response effect of LPS in the production of IL-6. Additionally, it gives the impression that the potency of *Bacteroides* LPS is greater than 100 fold less potent in comparison to the *E. coli* K12 LPS (Figure 4.22).

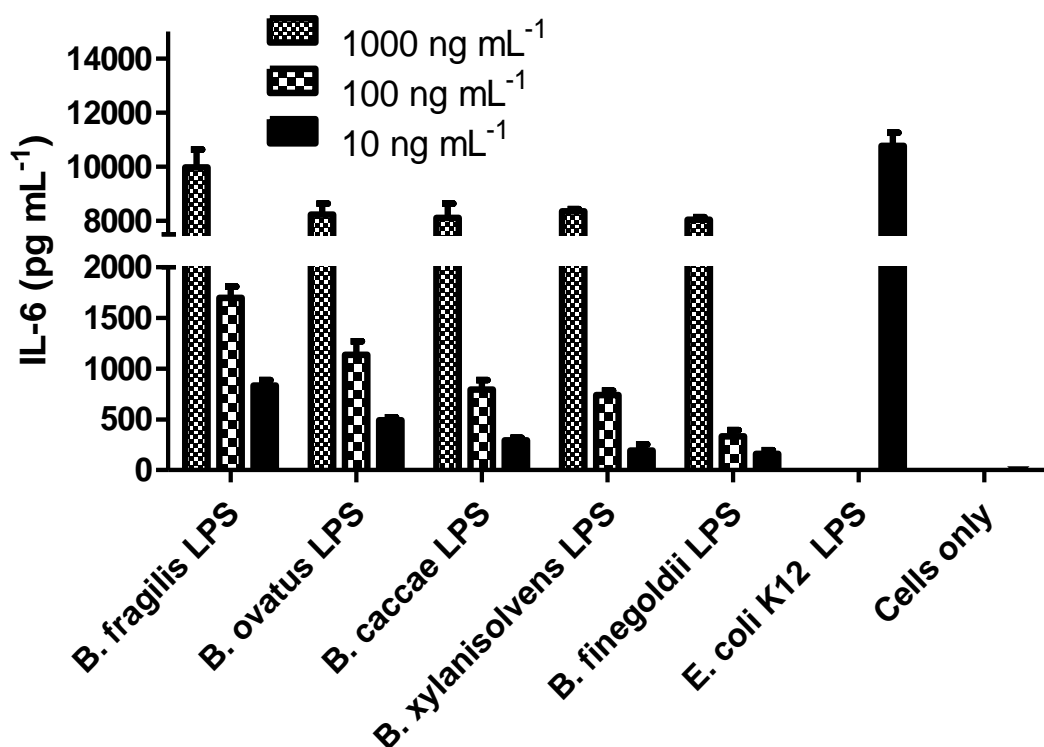


Figure 4.22: Dose response of lipopolysaccharide (LPS) induced IL-6 production from stimulated MPI cells measured using sandwich ELISA. Cells were stimulated with 10, 100, and 1000 ng mL⁻¹ of five *Bacteroides* species extracted LPS and 10 ng mL⁻¹ of *E. coli* K12 LPS was used as a positive control. Bars represent the mean and standard error of 3 independent experiments.

4.3.5.2 Immunological activity of *Bacteroides* LPS in MM6 cell line

Results indicated a dose response effect of LPS in the production of IL1-6 in the MM6 cell line. In addition, it seems that the potency of *Bacteroides* LPS is more than 100 fold less potent in comparison to the *E. coli* K12 LPS (Figure 4.23).

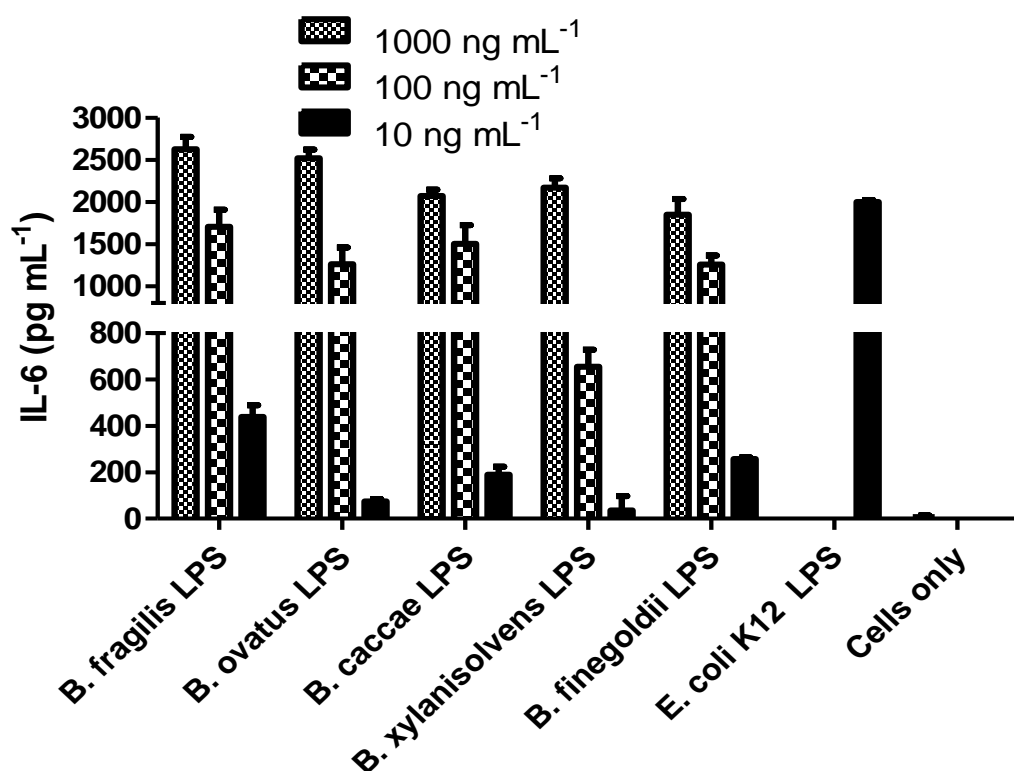


Figure 4.23: Dose response of lipopolysaccharide (LPS) induced IL-6 production from stimulated MM6 cells measured using sandwich ELISA. Cells were stimulated with 10, 100, and 1000 ng mL⁻¹ of five *Bacteroides* species extracted LPS and 10 ng mL⁻¹ of *E. coli* K12 LPS was used as a positive control. Bars represent the mean and standard error of 3 independent experiments.

Results showed a dose response effect of LPS in the production of TNF- α in the MM6 cell line. In addition, it seems that the potency of *Bacteroides* LPS is more than 100 fold less potent in comparison to the *E. coli* K12 LPS (Figure 4.24).

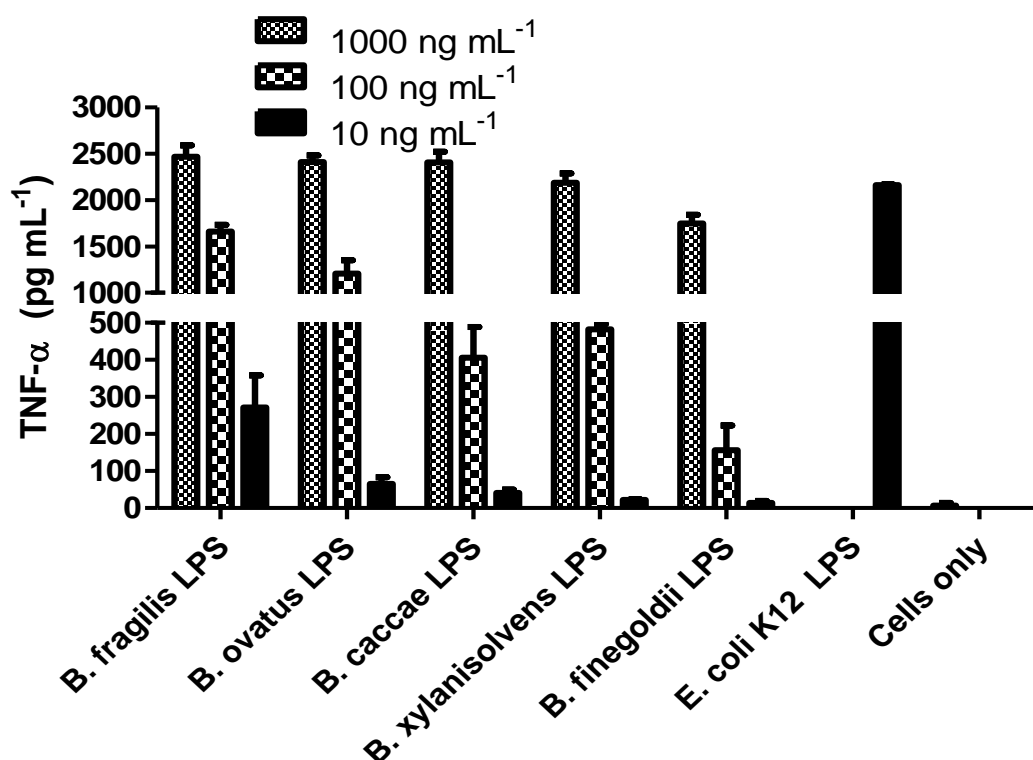


Figure 4.24: Dose response of lipopolysaccharide (LPS) induced TNF- α production from stimulated MM6 cells measured using sandwich ELISA. Cells were stimulated with 10, 100, and 1000 ng mL⁻¹ of five *Bacteroides* species extracted LPS and 10 ng mL⁻¹ of *E. coli* K12 LPS was used as a positive control. Bars represent the mean and standard error of 3 independent experiments.

4.3.6 ELISA assay to detect *Bacteroides* LPS using anti-*Bacteroides* LPS antibody

In this chapter, a method was designed to detect whole cells and extracted LPS of *Bacteroides* as a potential biomarker of recent faecal bacterial contamination using two format of ELISA technique: sandwich and direct ELISA as explained in the materials and methods section. Unluckily to what was expected, both methods showed high levels of nonspecific binding even with negative controls (LAL water only) after several attempts of each. In order to further investigate

the whether the antibody was functioning, different format of dot blot assay were conducted. Dot blot results showed that there was no interaction between the antibodies (unconjugated and biotin conjugated anti *Bacteroides* LPS antibody) and the LPS. *Bacteroides* LPS was incubated with the anti-*Bacteroides* antibody, followed by assessing the LPS activity using LAL assay. The levels were similar between LPS incubated with the antibody and LPS alone indicating that there was no interaction between the *Bacteroides* LPS and the anti-*Bacteroides* LPS antibody.

4.4 Discussion

In this chapter, *Bacteroides* species isolated from Challaborough beach were investigated for a future more specific faecal indicator by targeting their LPS as a biomarker. Pure cultures of *Bacteroides* species were isolated and speciated using phenotypic and genotypic methods in addition to knowing their source of host origin. In summary, five *Bacteroides* species isolated from marine bathing water samples and identified to be from a range of sources including human. Profiling of extracted LPS from these five *Bacteroides* species using SDS-PAGE successfully showed high similarity in LPS banding pattern mainly rough type across the *Bacteroides* species. In addition, the LAL activity of LPS from *Bacteroides* species was investigated and results were highly similar across the five identified *Bacteroides* species and very different from *E. coli* LPS reference activity. The biological activity of the extracted LPS from five *Bacteroides* species in cell culture models showed similar dose response trend across *Bacteroides* species with low potency in stimulation and production of

proinflammatory cytokines in comparison to *E. coli* LPS reference. These results show that the LPS from isolated *Bacteroides* shares similar characteristics and may possibly be developed as a faecal-specific biomarker in marine bathing waters. An ELISA assay was designed to specifically target the LPS of *Bacteroides* species using a commercially available anti-*Bacteroides* LPS antibody, however, the assay was unsuccessful because of non-functional antibodies. This ELISA method has a high potential and require further investigation in the future work.

Water samples collected from the stream (study area) were assessed for host-specific *Bacteroides* 16S rRNA genetic biomarkers using three previously described primer sets (universal *Bacteroides*, human and cow) and a fourth set recently described dog-specific *Bacteroides* 16S rRNA genetic biomarker primer set (Hussein *et al.*, in press). Positive results for all biomarkers in all three water samples were observed. Although this beach is designated as a dog-free beach, biomarkers of dog faeces were detected in the stream which pours directly into the bathing area. In addition, although the presence of dog and cow biomarkers in these waters raises the issue of faecal contamination, the presence of human faecal biomarker is more critical as it can lead to health problems. This faecal contamination could be due to the runoff of the surrounding area or failing septic systems. Since results were positive in all 3 seawater samples using Dog, Cow and Human *Bacteroides* host-specific primer sets, and since only one sample was collected at a storm event while the other two samples collected in calm weather indicating that the presence of human biomarker is weather-independent and could be possibly due to septic system of the surrounding area

near by the beach which the stream runs through and pours into the bathing area. Hence, *Bacteroides* species that are present in the bathing water in that particular beach comes from different hosts. Returning to the speciation of *Bacteroides* species, A hundred and one *Bacteroides* marine isolates were initially speciated using classical biochemical tests (described in Bergey's Manual of Systematic Bacteriology) and five *Bacteroides* species (*B. caccae*, *B. ovatus*, *B. xylanisolvens*, *B. fragilis* and *B. finnegoldii*) have been identified followed by confirmation using molecular methods. Downstream analyses were conducted on these *Bacteroides* species to explore their similarity in structure. A follow up experiment to chapter three was conducted where the LAL activity of LPS from *Bacteroides* species was investigated and showed high similarity across the *Bacteroides* species, however, their activity was <300 folds less than a *E. coli* (O55:B5) reference LPS. It has been widely known that the LPS from *E. coli* is most active LPS form (Zughaier *et al.*, 1999a), Hence, results suggest that the increase in total LPS activity in contaminated bathing waters could be attributed mainly to *E. coli* LPS.

Although *Bacteroides* numbers correlated highly to the total LPS in collected water samples (Figure 3.11 B) they were not the main activators in the LAL assay because of their low active LPS. Similarly, the potential biological relevance of extracted lipopolysaccharide of the five *Bacteroides* species showed a similar dose response trend, however, their potency was found to be at least 100 times less than *E. coli* K12 reference LPS. This agrees with the findings of Lindberg (1990) and colleagues who observed that *Bacteroides fragilis* is 100-1000 fold less potent than *E. coli* or *Salmonella* in the

Shwartzman reaction. This could be mainly due to penta acyl and one phosphate group in the *Bacteroides fragilis* in comparison to hexa acyl and dual phosphate groups (Rietschel *et al.*, 1994). It has been thought that exploring the immunogenicity of *Bacteroides* species present in marine bathing water was important to be conducted because one of the main criteria of a bacterial indicator or a biomarker is that it must be non-pathogenic and has no or little effect on human health. In addition, because there are no regulations in the current European directive regarding the levels of LPS in marine bathing water, hence, this study investigated the immunogenicity of extracted LPS from *Bacteroides* marine isolate using two cell culture models. When LPS enter the human body they stimulate inflammatory reactions such as the release of cytokines from monocytes, neutrophils, and vascular endothelial cells. These cells interact with LPS binding protein (LBP) which transfers LPS to CD14 that is present as a soluble form or on the cell surface of macrophages, monocytes and dendritic cells. The CD14 then transfers it to MD2 TLR4 complex which initiates a cascade of intracellular molecule activation and leading to cellular signalling and cytokine release. Although the LAL assay activity showed very similar results among *Bacteroides* and the LPS profiling showed high similarity, however, they were not identical, hence, possibly a difference in one or more sugar molecule could make a difference in LPS cellular signalling and subsequently cytokine production. In addition, researchers still argue that *Bacteroides* LPS might signal through TLR2 instead of TLR4; the usual classical pathway for LPS cell signalling (Erridge *et al.*, 2004, Alhawi *et al.*, 2009, Round *et al.*, 2011). This may well be true and require further investigation.

Since *Bacteroides* showed a high correlation to the total LPS levels (results from chapter three) a method was designed that employs *Bacteroides* LPS as a biomarker for faecal indicators. A thought of using ELISA assay was attempted after investigating the different *Bacteroides* species LPS profile. Results showed a high similarity among the *Bacteroides* species LPS banding pattern indicating a potentially promising candidate to be used as future faecal indicator biomarker.

SDS-PAGE profiling of LPS extracted from *Bacteroides* species showed high similarity (>95%) in banding pattern; and less than 10% when compared with profiled LPS from *E. coli* LPS isolated from marine bathing water samples. LPS profiling showed a typical rough LPS banding pattern while *E. coli* LPS showed a classical ladder-like smooth LPS. The reason behind choosing to extract LPS from *Escherichia coli* strain U 5/41 (sequence in the appendix D) isolated from seawater that was used as a reference for comparison when profiling *Bacteroides* was to demonstrate two points: the first point was to assess whether *E. coli* present in seawater possess different LPS to *Bacteroides* LPS since all the bacterial and *E. coli* isolates were isolated from bathing water, it was thought best to use indigenous bacteria to compare the LPS banding pattern with in case of changing in LPS characteristics due to environmental stresses (Tao *et al.*, 1992). The second point was to assess if the extraction method might have an effect on the LPS, i.e. commercially extracted LPS might have been extracted and purified differently. Different LPS extraction methods yields different LPS banding pattern based on influencing the chemical composition of the product, different biological activity of the different LPSs, in

addition that different growth media influence the chemical composition and/or the biological activity of LPS. Our intention was to determine the overall properties of different *Bacteroides* species extracted LPS and show similarity of LPS pattern across them. In addition, it has been showed that extraction of LPS from the same bacterial species using different extraction method was different (Poxton and Edmond, 1995). Hence for standardisation measures, the author sought to extract the LPS of *Bacteroides* and *E. coli* using the same LPS extraction method.

Since *Bacteroides* showed a high correlation to the total LPS levels (results from chapter three) a method was designed that employs *Bacteroides* LPS as a specific biomarker for faecal indicators. Sandwich ELISA assay was designed attempting to detect the *Bacteroides* LPS using anti-*Bacteroides* LPS antibody. Since the Sandwich ELISA assay failed to work after several attempts even after using higher concentrations of LPS, capture and detection antibodies in addition to a several attempts with whole *Bacteroides fragilis* bacterial cells, a direct ELISA was attempted. Both ELISA assays were unsuccessful to detect the *Bacteroides* LPS because the antibody failed to work showing high levels of non-specific binding after several trials. Hence, a troubleshooting methods were conducted to assess the specificity of both unbound and biotinylated anti *Bacteroides* LPS antibodies. However, all endeavours to assess the functionality of the anti-*Bacteroides* LPS antibodies were unsuccessful. Results also showed that there was no difference between LPS incubated with the antibody and LPS without the antibody (control) which suggests that the anti-*Bacteroides* LPS antibody had no interaction/binding with the LPS and had no

effect on the reactivity of LPS in the LAL assay. The commercial provider for anti-*Bacteroides* LPS antibody was contacted and asked to provide information regarding the source of the *Bacteroides* LPS used as an immunogen, the exact ELISA protocol used to test this antibody and the extraction method of LPS used as a standard, however, the response to all of the above questions was “we do not have any information regarding your inquiries”. Hence, after all the endeavours to test this antibody, it was difficult to pin point how to resolve this problem and reached a conclusion that the best practice to tackle this problem is to raise antibody specific to the extracted *Bacteroides* LPS (future work).

In conclusion, water samples collected from the stream that pours into the bathing area of Challaborough beach were tested for three host specific *Bacteroides* biomarkers and showed to be positive for all three biomarkers (human, dog and cow). Further work needs to be conducted to further investigate and possibly apply remedies to eliminate the source of this faecal contamination and prevent possible contamination of the beach with potentially pathogenic microbes. So far, however, there have been no studies conducted to explore the effects of contaminated bathing waters on human health. The *Bacteroides* species were successfully profiled and identified using culture based methods and confirmed with biochemical and molecular technologies. Extracted LPS of these *Bacteroides* isolates was successfully profiled and statistically shown to have a high similarity in banding patterns and endotoxic activity in all *Bacteroides* isolates extracted LPS. When the LPS LAL activity of the five *Bacteroides* species was compared to *E. coli* O111:B4 LPS, the LPS from *E. coli* was 347 fold more active than *Bacteroides* species. Since the

Bacteroides species possess only one phosphate and five acyl groups compared to two phosphate and 6 acyl groups in *E. coli* (Rietschel *et al.*, 1994) radically decreases the potency of LPS and the LAL activity (Takada *et al.*, 1988) and explains the low potency in cell culture models in comparison to *E. coli* LPS reference. Nonetheless, this indicates that *Bacteroides* LPS from seawater, even with varying speciation may have similar potency. Although *Bacteroides* participates as a small quantity of the total LPS it correlates to total LPS and to other bacterial indicators and this highly supports the notion of monitoring *Bacteroides* as more indicative for recent faecal contamination. Since *Bacteroides* showed the highest correlation to total LPS (Pearson correlation of LPS and *Bacteroides* = 0.954 $p < 0.001$), a LAL assay was performed to determine how much *Bacteroides* contributed to the total endotoxic activity in water samples. 2.7×10^3 *Bacteroides* cells, which represent the highest number, were recorded in the shallow bathing water using culture based methods and were assayed at 0.06 EU mL⁻¹ for their total LPS activity. *Bacteroides* specific LPS in seawater were profiled for future development of this as a specific biomarker and showed high similarity in the banding pattern. Finally, an ELISA method was designed to detect the LPS of *Bacteroides* species, however, the antibodies ordered seemed non-functional, this method is very promising and a follow up work will be conducted in the near future.

The bacterial composition might vary from one beach to another; this is also true to the presence of *Bacteroides* species present in marine bathing waters. Various factors might lead to variation in the *Bacteroides* community present in such waters; this might include type of human diet, temperature and source of

Bacteroides (Bradley *et al.*, 1999) Hence, similar studies are required to be conducted on different study areas (beaches) to investigate the prominent *Bacteroides* presence.

So far, however, there have been no studies conducted to explore the biological activity of contaminated bathing waters. Chapter five sheds light on the potential biological relevance of contaminated bathing seawater on human health used to stimulate cell culture models and measure the release proinflammatory cytokines.

**Chapter 5: Evaluation of inflammatory effects of
contaminated marine bathing water using *in vitro* cell
culture models**

5.1 Introduction

A considerable amount of literature has been published that associated polluted marine water with various human (bathers') health pathogens (Cabelli, 1983, Cabelli, 1989, Cabelli *et al.*, 1979, Cabelli *et al.*, 1982, Cabelli *et al.*, 1983, Cheung *et al.*, 1990). These health problems include incidence of marine-related illnesses after exposure to marine water. Several factors play a key role in developing water-related illnesses such as the length and type of water recreational activities (Corbett *et al.*, 1993), presence of pathogens and their concentration (Cheung *et al.*, 1990, Kay *et al.*, 1994), and immune status of the bathers. The microbial quality of recreational waters in Europe is governed by the European Bathing Water Directive 2006/7/EC, but this Directive is still not suitable for prediction of health risks of waterborne diseases from recreational marine waters due to the drawbacks of conventional culture based methods used to monitoring the quality of marine bathing waters. One of the main drawbacks can be due to the persistence of some pathogens (especially viruses) for longer periods than faecal indicators. Hence, enumerating the levels of FIB will underestimate the quality of marine bathing water. In addition, certain sea sediment types are regarded as a reservoir of bacteria (Mueller-Spitz *et al.*, 2010); a potential sanctuary for such pathogens which could be dispersed in the water column in the event of storms and become infective whenever suitable conditions present. The source of enteric pathogens present in recreational marine waters could result from poorly treated faecal pollution from sewage discharge or from runoff from farms of the surrounding areas (Crossette, 1996).

Several studies have attempted to define the levels of health risks following human exposure to different concentrations of pathogens and indicator organisms in recreational waters (Cabelli, 1983, Cabelli, 1989, Cabelli *et al.*, 1979, Cabelli *et al.*, 1982, Cabelli *et al.*, 1983, Cheung *et al.*, 1991, Fleisher *et al.*, 1993, von Schirnding *et al.*, 1992).

All these studies were epidemiological surveys conducted by recruiting individuals at beaches who have been involved in water activities. In addition, individuals who conducted no water activities were also recruited as a control group. After a few days, a remote follow up procedure took place with the participants documenting if any illness has developed. Although any illnesses could be due to swimming in marine bathing waters, they could be also due to eating, for example, contaminated sandwiches on the beach or an ice cream, etc. as confounding factors (Kay *et al.*, 1994). Kay's study has shown that swimmers acquired illness on days when bacterial indicator levels were acceptable (Kay *et al.*, 1994). Kay's study also supports the notion that faecal indicator bacteria alone are inadequate surrogates for pathogen presence in marine bathing waters. These illnesses could be due to presence of high levels of LPS.

When LPS enters the blood stream of humans, even picograms amounts are capable of causing a potent immunological reaction (Rice *et al.*, 2003). LPS signals through Toll-like receptor 4 (TLR4, pathway illustrated in figure 1.4) and stimulates the release of inflammatory mediators including cytokines in monocytes, neutrophils, and vascular endothelial cells. This can cause a variety

of symptoms ranging from high fever, tissue injury, and in severe cases death by multi organ failure (Shapira *et al.*, 1996).

LPS enters the human body via skin breaks resulting from scratches, cuts, wounds, or abrasion (Kindt *et al.*, 2006). In addition, LPS can cause ocular pathology after entering through damaged corneas (Schultz *et al.*, 2000). Furthermore, it has been suggested by Sandstorm (1992) that inhalation of LPS can cause transient or chronic lung function impairment. Although LPS was considered harmless in the intestine providing protective effects against epithelial injury, it has been suggested that LPS might induce intestinal inflammation, even necrotising enterocolitis (Im *et al.*, 2012). Ingestion of bathing water containing LPS while swimming is also a possible route to entering blood stream. Kiefer and Ali-Akbarian (2004) hypothesised that LPS might be one of the causes of a disruption in the tight junctions of intestinal epithelial cells leading to malfunction and increase in gut permeability. Increasing of gut permeability can lead to the translocation LPS from the gut into the blood stream and cause various immunological reactions.

Inhalation of bacteria present in aerosols of marine seawater is also a possible route of infection and it is via bacterial emissions (Hultin *et al.*, 2011). LPS can be aerosolised by wave action causing a potential respiratory tract exposure and respiratory asthma-like symptoms, especially in immunocompromised individuals and children (Coye and Goldoft, 1989). In addition, LPS might access the blood stream through openings in the genitourinary tract and sepsis cannot be excluded. LPS can elicit irritant and allergenic responses in human and animal tissues (Sivonen and Jones 1999), also they are pyrogenic and toxic

(Weckesser and Drews 1979). In addition, it has been suggested that an outbreak of gastroenteritis in Pennsylvania in the United States may have been caused by cyanobacterial LPS (Lippy and Erb 1976). To the best of our knowledge, this was the first study conducted to assess the biological effect of contaminated bathing waters *in vitro* using cell culture models by measuring proinflammatory cytokines. “Contaminated” and “clean” bathing water samples, based on the European Union water directive indicators levels (2006), were used as stimulants in cell culture models to investigate their potential biological relevance. Key immune cells that are involved in inflammation, monocytes and macrophages, are highly responsive cell types for LPS signalling both *in vivo* and *in vitro* (Fejer *et al.*, 2013, Liu *et al.*, 2011). Previous studies have shown that MM6 cell line is a good human blood monocytes model to study the potential biological relevance of LPS (Liu *et al.*, 2011, Zughaier *et al.*, 1999a, Zughaier *et al.*, 1999b, Bamford *et al.*, 2007). In addition, Mouse alveolar-like macrophage MPI have been shown to be a good *in vitro* model to investigate pathogenesis of lung inflammation and test production of proinflammatory cytokines by LPS (Fejer *et al.*, 2013).

In the current study, it has been aimed to use the cultured mouse macrophages MPI cells and human monocytic cells MM6 as *in vitro* models to test whether LPS present in marine bathing water samples induce production of proinflammatory cytokines interleukin TNF- α IL-1 α , IL-6 and IL-8. To assess whether the possible cytokine-stimulating effect of water samples is predominantly attributable to LPS, a LPS inhibitor, Polymyxin B (PMB), was

used in our studies as previously described (Becker *et al.*, 2005, Liu *et al.*, 2011).

5.2 Materials and methods

5.2.1 Water samples preparation

In order to investigate the biological effects of LPS present in “clean” and “contaminated” bathing waters, tenfold serial dilutions of four seawater samples were prepared to be used as stimulant in cell culture models. These water samples were selected from the collected shallow bathing water samples over the summer of 2012 where the number of bacterial indicators and LPS levels are known. These water samples represent “highly contaminated”, “contaminated”, “on the threshold” and “clean” bathing water samples in relation to the European Union bathing water directive regulation. Water sample criteria are detailed in table 5.1 and they are referred to thorough out this chapter:

Table 5.1: Criteria and type of bathing water samples* selected as stimulants in cell culture models.

Water samples	<i>E. coli</i> (CFU 100 mL ⁻¹)	<i>Bacteroides</i> (CFU 100 mL ⁻¹)	LPS activity (EU mL ⁻¹)
“Highly Contaminated” water	4535 ± 150	2720 ± 68	230 ± 0.40
“Contaminated” water	2742 ± 84	1728 ± 52	135 ± 0.35
“S. bathe” water (threshold level)	560 ± 26	377 ± 18	58 ± 0.28
“Clean” water	70 ± 12	18 ± 2	6 ± 0.30

*Numbers represent mean value of four samples, \pm standard deviation of the mean.

5.2.2 Cell culture

Two cell culture models were used to investigate the biological effect of LPS present in “contaminated” and “clean” bathing waters. Maintenance of MM6 cells and MPI were described in the general materials and methods section (Chapter 2).

5.2.3 Stimulation of MM6 cell line with bathing water samples

MM6 cell line was used in this study to evaluate the inflammatory effects of bathing water samples. MM6 cells were stimulated with water samples, then the release of proinflammatory cytokines was measured using sandwich ELISA. MM6 cells were maintained as described in chapter two. MM6 cells were seeded in 48 well plates giving a final concentration of 1×10^6 cells mL^{-1} (Nunc, Fisher Scientific, UK). Serial dilutions of water samples (1/10, 1/100 and 1/1000) were prepared in LPS-free water and used to stimulate MM6 cells. Similar dilutions were prepared and pre-incubated with $10 \mu\text{g mL}^{-1}$ Polymyxin B sulphate (PMB) (Sigma, St. Louis, MO) with water samples or an *E. coli* K12 LPS standard, leaving only the biological effect of any non-LPS stimulants present in the water samples. In similar studies, dose response experiments showed that $10 \mu\text{g mL}^{-1}$ completely inhibited the effect of LPS (Becker *et al.*, 2005, Cardoso *et al.*, 2007). Positive control *E. coli* LPS, PMB alone, and RPMI

medium only to untreated cells were also included in each experiment. Cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere for 24 hours. Four proinflammatory cytokines; TNF- α , IL-1 α , IL-6, and IL-8 were assessed using human Milliplex multianalyte panel (xMAP, Millipore, UK). Supernatant was collected after the proper incubation period by aspirating the well content into 1.5 mL sterilized microcentrifuge tubes then spun at 160 x g for 5 minutes. Supernatant was then transferred into 2 microcentrifuge tubes and kept at -20 °C until assayed. *E. coli* K12 purified LPS (Sigma, UK) was included in this study as a reference standard (Positive control) since the LPS from *E. coli* is often reported to represent the most biologically active form of LPS (Rietschel *et al.*, 1992).

5.2.4 Stimulation of MPI cells with bathing water samples

MPI are newly described self-renewing, GM-CSF dependent non-transformable lung alveoli macrophage like cells (Fejer *et al.*, 2013). MPI cells were used in this study to evaluate the inflammatory effects of bathing water samples. Since the MPI cells have the characteristics of alveoli macrophage, it could shed light on the immunological responses in the lung. Cells were maintained as described in chapter two. In all experiments conducted, the viability of cells was more than 97%. Cells were seeded in each well of a 96 well plate giving a final concentration of 1×10^5 cells per well. MPI Cells were stimulated with the same serial dilutions of water samples (1/10, 1/100 and 1/1000) that were used to stimulate MM6 cells. Similar dilutions were prepared and pre-incubated with 10 $\mu\text{g mL}^{-1}$ Polymyxin B sulphate (PMB) (Sigma, St. Louis, MO) with water samples or an *E. coli* K12 LPS standard, leaving only the biological effect of any

non-LPS stimulants present in the water samples. Positive control *E. coli* LPS, PMB alone, and RPMI medium only to untreated cells were also included in this study. Cells were incubated at 37 °C in a humidified 5% CO₂ atmosphere for 8 hours for TNF- α and 24 hours for IL-6 and IL-1α. Supernatant was collected after the proper incubation into 1.5 mL sterilised microcentrifuge tubes, and then spun at 160 x g for 5 minutes. Supernatant was then transferred into 2 microcentrifuge tubes and kept at -20 °C until assayed.

5.3 Detection of cytokines using sandwich ELISA and Milliplex human multianalyte panel cytokine assay

5.3.1 IL-6, TNF-α and IL-1α sandwich ELISA for MPI mouse macrophage cells

Methods were exactly followed as explained in section (4.2.11.6.4).

5.3.2 Milliplex human multianalyte panel cytokine assay

Milliplex human multianalyte panel is a technique used to simultaneously analyse multiple analytes in a single well by multiplexing using xMAP[®] technology thereby saving sample volume and time. Milliplex human multianalyte panel technique involves colour coded magnetic microspheres coupled with antibody specific to the analyte of interest. These microspheres are colour coded with 2 fluorescent dyes with precise ratios. One hundred coloured microspheres can be created, each of which can be coupled with a

specific antibody. After analyte is captured by a specific antibody, a biotinylated detection antibody is introduced. A streptavidin PE fluorescent reporter conjugate is added to report a signal of analyte presence. A sample probe aspirates well contents and allows individual beads to pass rapidly through a 2 laser detection system; one excites the microsphere dye in order to identify the bead depending on the 2 colours ratio, and the other excites the PE reporter which is converted into a quantitative protein concentration. A high-speed digital signal processor identifies each individual microsphere and quantifies the results of the analyte depending on the PE signal.

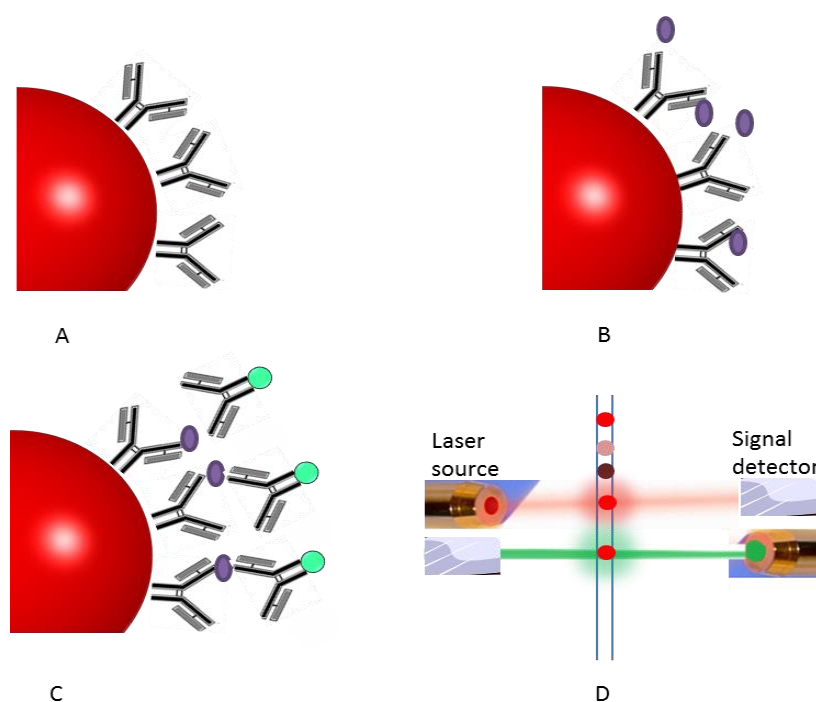


Figure 5.1: a schematic diagram of Luminex® xMAP multiplex™ principle (A) magnetic microsphere dyed with a distinctive fluorescent dye and coated with Ab specific to analyte of interest, (B) analyte of interest binds to specific antibody, (C) fluorophore-labelled secondary antibody binds to the analyte of interest, (D) Luminex® analyser laser detecting system identifies fluorescently labelled magnetic microsphere and quantifies analyte concentration labelled with secondary antibody.

Prior to commencing the experiment, maintenance routine procedure was carried out to calibrate the Luminex® multiplex™ analyzer (Luminex® 200™) by

selecting the maintenance option from the Luminex® xPONENT version 3.1 software's home page. Initially, sample probe height was adjusted to ensure that the sample probe drops enough into the well to acquire samples, this step is essential to guarantee a smooth run and avoid introducing air bubbles if the sample probe is higher than the sample level. This step was performed by selecting the drop sample probe, which allows the sample probe to drop into the well which is loaded with standard two large alignment discs (5.08 mm in diameter) that mimics the sample presence in wells. The sample probe was then lessened and gently forced to touch the top of the alignment discs then tightened again using the provided sample probe screw. Calibration and performance verification kits (Millipore, UK) were used to calibrate and optimise the Luminex® multiplex™ analyser. All reagents were allowed to warm up to room temperature before loading into the automated maintenance plate (Figure 5.2) and select the system initialisation procedure selected, this involves warming the analyser and laser optic system up in addition to running the calibration and verification assays. For the purpose of running multiplex cytokine quantification, 200 µL of wash buffer was added into each well of an opaque flat bottom 96 well plate and then the plate was placed on a plate shaker (500 rpm) at room temperature for 10 minutes. Washing buffer was discarded, then 25 µL of a fivefold standard range from 10,000-3.2 pg mL⁻¹, blank (RPMI 1640 only) and control 1 and 2 in addition to the cell culture supernatant samples were added in duplicate in addition to 25 µL of assay buffer to each well. TNF-α, IL-1α, IL-6 and IL-8 coupled microspheres were sonicated for 30 seconds then vortexed for 1 minute to assure no clumping then 60 µL of each vial were taken and pooled into a mixing bottle (provided with the

kit) and then the total volume brought to 3 mL. The mixing bottle was vortexed for 1 minute and 25 μ L of the pooled suspension added to each well. The plate was sealed and with a plate sealer and incubated overnight at 4 °C. After incubation, the plate was placed on a hand held magnetic separator in order to keep the microsphere inside the plate's wells and facilitate decanting of the well contents. After decanting well contents, the plate was washed twice with washing buffer, and then 25 μ L of detection antibody was added. Following this, the plate was sealed and left on a plate shaker for 1 hour at room temperature. 25 μ L of streptavidin-phycoerythrin was added to each well then again sealed and agitated on the plate shaker for 30 minutes. A washing step was carried out as described above, then 150 μ L of sheath fluid was added to all wells then the plate was left on the plate shaker for 5 minutes. Finally, the plate was run on the Luminex[®] 200 analyser with xPONENT 3.1 software.



Figure 5.2: (A) picture of Luminex[®] multiplex[™] analyser (Luminex[®] 200[™]). (B) picture of Automated maintenance plate which is used to calibrate the Luminex[®] multiplex[™] analyzer, red arrows represent where calibration and verification kits' reagent added in a particular order, green arrow shows the large alignment disc used for sample probe adjustment. Distilled water, 70% ethanol and 10% bleach added for washing, flushing and sanitation steps respectively.

5.4 Statistical analysis

One way analysis of variance (ANOVA) and Tukey's HSD was carried out using GraphPad prism version 5. Four parameters logistic ELISA standard curves were constructed using softmax pro version 5.4. Five parameters logistics Magplex Luminex[®] were constructed using xPONENT 3.1 software. Samples were run in duplicate (technical replicate) and three independent experiments (biological replicates). A $p < 0.05$ was considered significant.

5.5 Results

5.5.1 Results of stimulating the MPI cells

5.5.1.1 IL-1 α secretion

The levels of IL-1 α induced by water samples from the MPI cells were assessed. Results showed that PMB at a concentration of 10 $\mu\text{g mL}^{-1}$ was adequate to inhibit the effect of *E. coli* K12 LPS and the levels of induced cytokines were similar to the levels of untreated cells. Cytokine induction showed no significant difference between unstimulated cells and cells stimulated with Instant Ocean (Underworld, UK), indicating that salinity has no effect on cell line stimulation.

"Highly contaminated" bathing water samples induced IL-1 α from MPI cells and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease ($p < 0.01$) in inducing IL-1 α , indicating that LPS was the main stimulant (Figure 5.3).

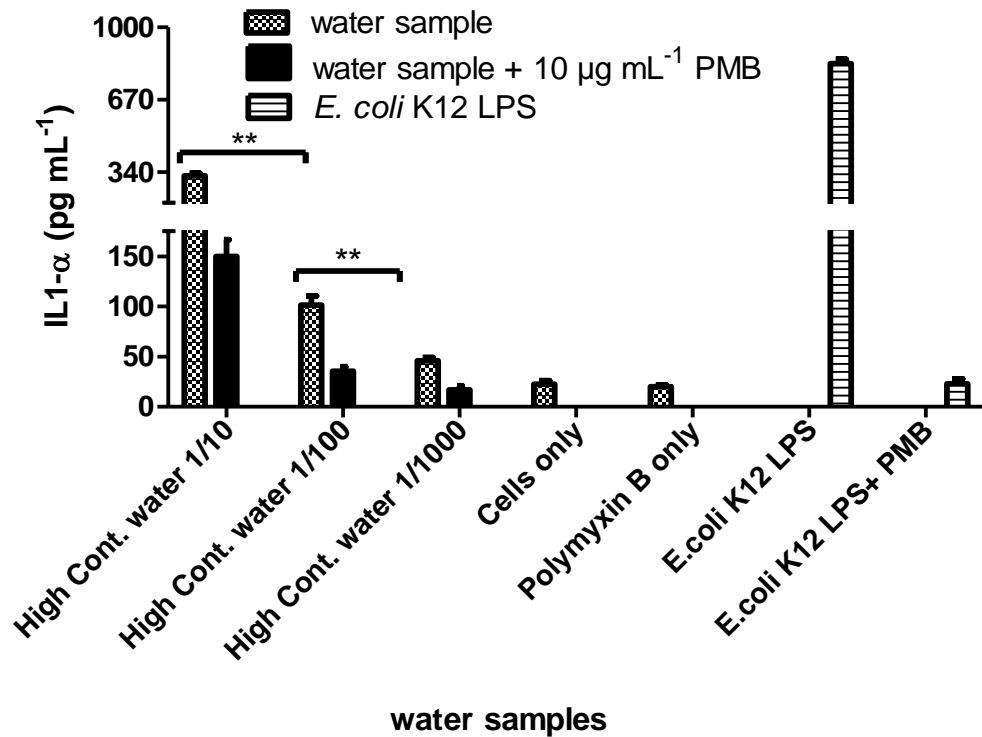


Figure 5.3: Effect of “highly contaminated” bathing water (high Cont. water, 230 EU mL⁻¹) samples on IL-1α cytokine secretion in the MPI cell culture medium measured using sandwich ELISA. Cells were treated with 1/10, 1/100 and 1/1000 dilution of water samples for 24 hours. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Error bars represent standard error of the mean of 3 independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences where (**) $p < 0.01$. *E. coli* LPS was included as a positive control.

Similarly, “contaminated” bathing water samples induced IL-1α from MPI cells and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease ($p < 0.01$) in inducing IL-1α indicating that the main stimulant was attributable to LPS (Figure 5.4).

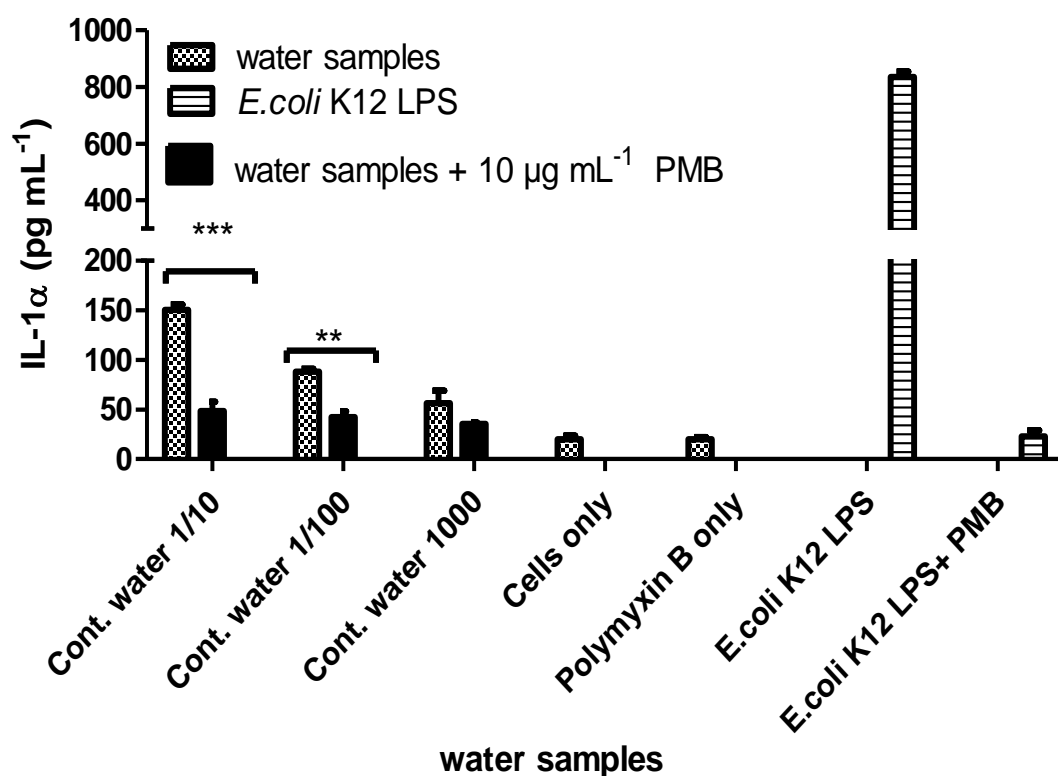


Figure 5.4: Effect of “contaminated” bathing water samples (Cont. water, 135 EU mL⁻¹) on IL-1α cytokine secretion in the MPI cell culture medium measured using sandwich ELISA. Cells were treated with 1/10, 1/100 and 1/1000 dilution of water samples for 24 hours. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Error bars represent standard error the mean of 3 independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences where (**) $p < 0.01$ and (***) $P < 0.001$. *E. coli* LPS was included as a positive control.

“S. bathe” water samples (slightly above the threshold of 50 EU mL⁻¹) induced IL-1α from MPI cells and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease ($p < 0.05$) in inducing IL-1α indicating LPS was the main stimulant (Figure 5.5).

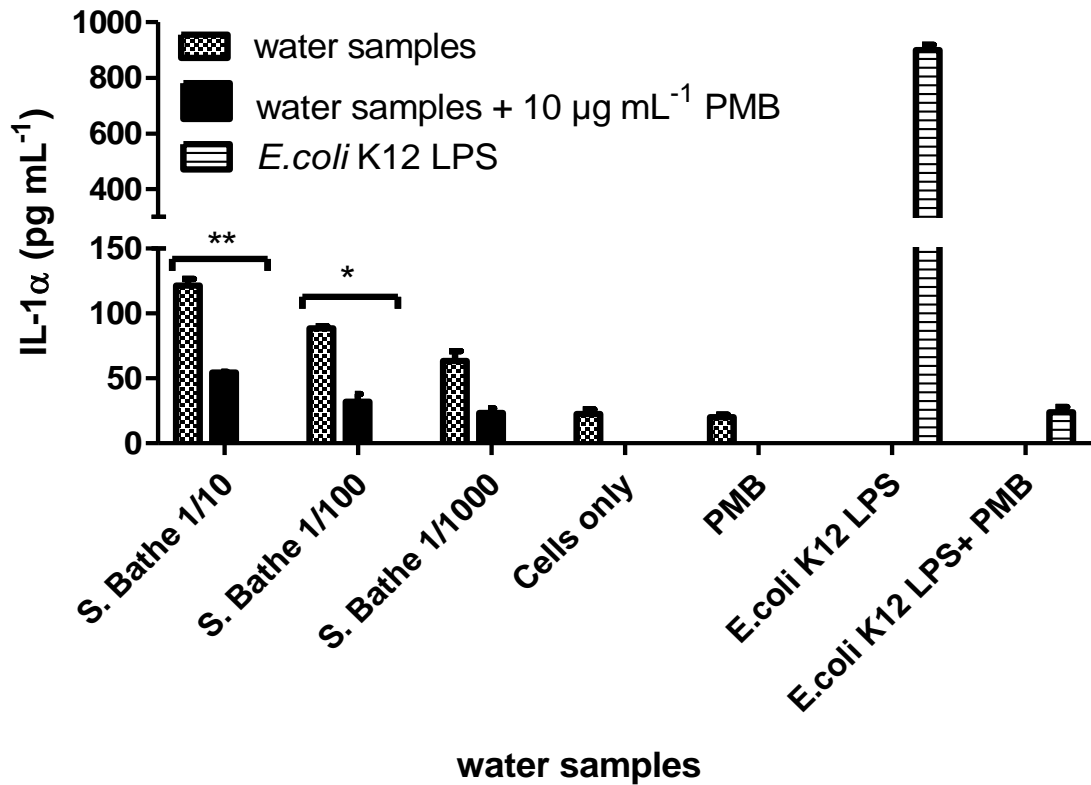


Figure 5.5: Effect of “S. bathe” water samples (58 EU mL⁻¹) on IL-1α cytokine secretion in the MPI cell culture medium measured using sandwich ELISA. Cells were stimulated with 1/10, 1/100 and 1/1000 dilution of water samples for 24 hours. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Error bars represent standard error of the mean of 3 independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences where (*) $p < 0.05$, (**) $p < 0.01$. *E. coli* LPS was included as a positive control.

“Clean” marine bathing water samples induced IL-1α from MPI cells and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease ($p < 0.01$) in inducing IL-1α indicating LPS was the main stimulant (Figure 5.6).

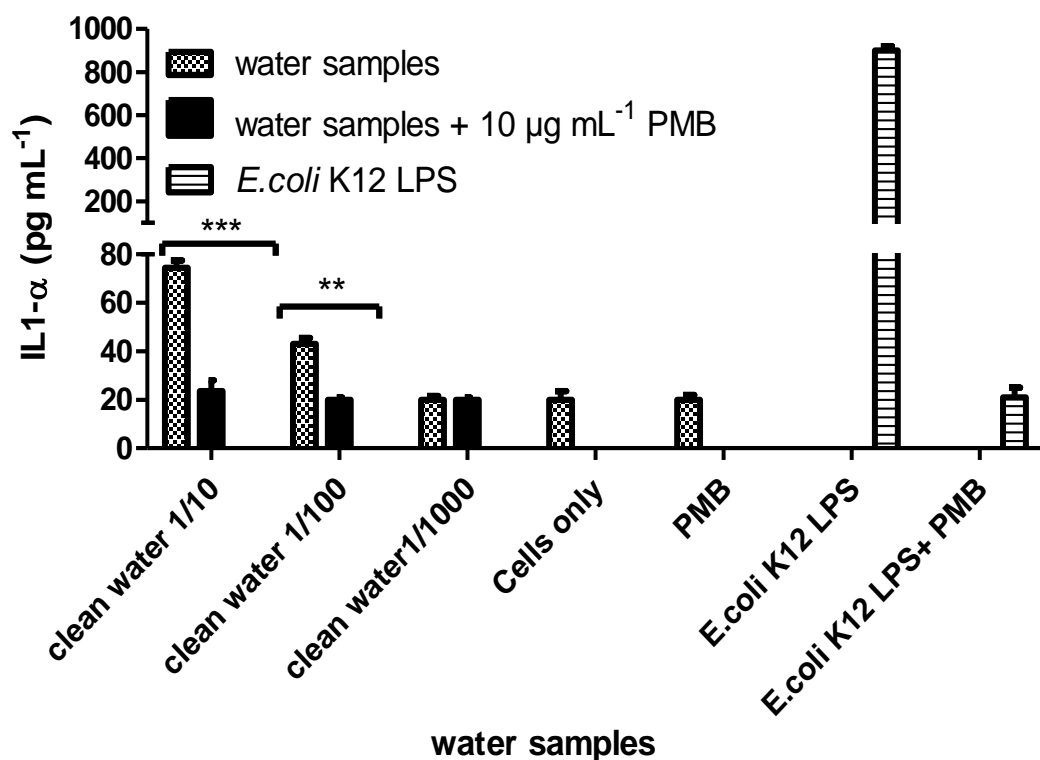


Figure 5.6: Effect of “clean” water samples (6 EU mL⁻¹) on IL-1α cytokine secretion in the cell culture MPI measured using sandwich ELISA. Cells were treated with 1/10, 1/100 and 1/1000 dilution of water samples. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Error bars represent standard error of the mean. Bars represent mean of 3 independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences where (**) $p < 0.01$ and (***) $p < 0.001$. *E. coli* LPS was included as a positive control.

As can be seen from graphs 5.3-5.6 above that the main stimulant in marine bathing water samples was due to the presence of LPS and the secretion of IL-1α showed a dilution effect. In order to show the significant differences between water samples in the stimulation of MPI cells and secretion of IL-1α, the levels of induced IL-1α from the least dilution (1/10 of each water sample) with PBM were subtracted from the least dilution (1/10 of each water sample) total IL-1α levels leaving only the effect of LPS. These levels were then converted into number of fold difference to the resting untreated cells (Figure 5.7 B). In

addition the total fold increase of IL-1 α without subtracting the non-LPS is also shown in figure 5.7 A.

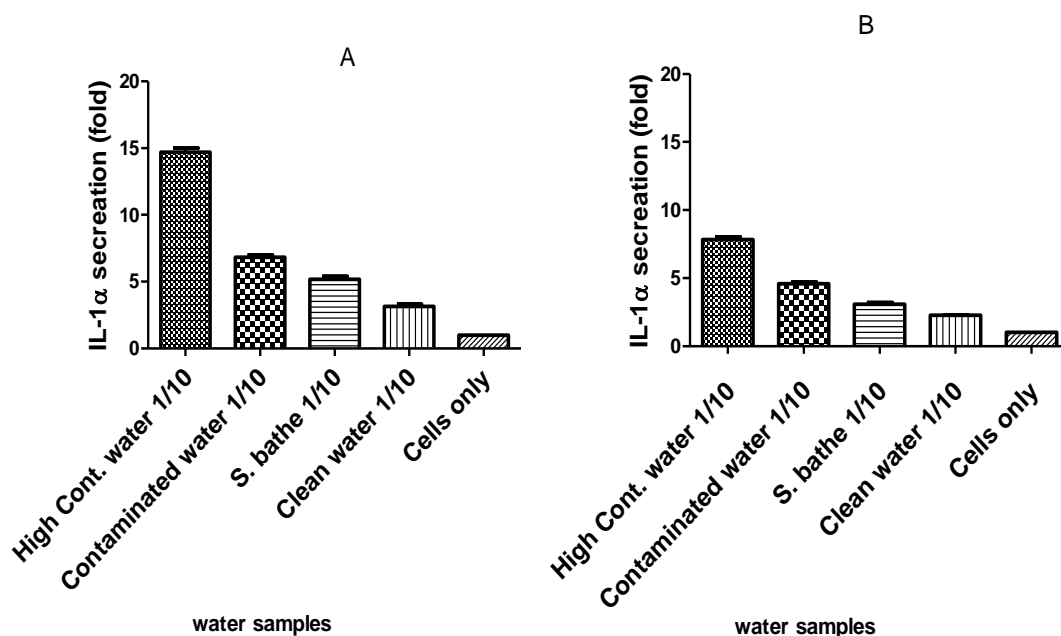


Figure 5.7: Effect of marine bathing water samples (High cont. water 230 EU mL⁻¹, “S. bathe” 58 EU mL⁻¹ and “clean” water 6 EU mL⁻¹) on the secretion of IL-1 α . Results are expressed as a number of folds increase compared to the control cells (A) represent the total fold increase of IL-1 α (LPS and non LPS stimulants) (B) fold increase attributed to LPS only after deducting the levels of non-LPS stimulants. Statistical analysis conducted using one way ANOVA and Tukey’s HSD was carried out to determine significant differences. All showed a significant difference of $p < 0.001$, except between “S. bathe” and “clean” water where $p < 0.01$.

This is clearly showing that “highly contaminated” bathing water induced the highest secretion of IL-1 α in comparison to “clean” bathing waters and that was mainly attributed to the LPS.

5.5.1.2 TNF- α secretion

TNF- α levels induced by water samples from the MPI cells were measured. Results showed that PMB at a concentration of 10 μ g mL⁻¹ were sufficient to

inhibit the effect of *E. coli* K12 LPS and the levels of induced cytokines were similar to the levels of untreated cells. Cytokine induction showed no significant difference between unstimulated cells and cells stimulated with Instant Ocean (Underworld, UK), indicating that salinity has no effect on cell line stimulation.

“Highly contaminated” bathing water samples induced TNF- α from MPI cells and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease ($p < 0.001$) in inducing TNF- α indicating LPS was the main stimulant (Figure 5.8).

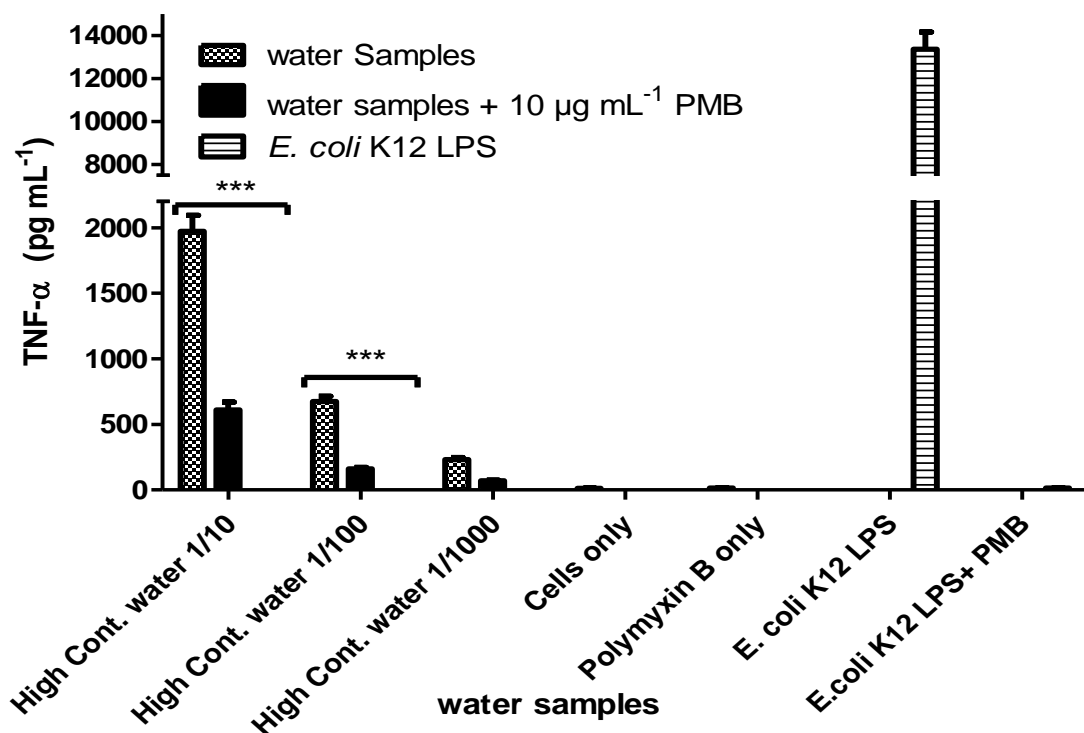


Figure 5.8: Effect of “highly contaminated” bathing water samples (High Cont. water, 230 EU mL⁻¹) on TNF- α cytokine secretion from the cell culture MPI measured using sandwich ELISA. Cells were treated with 1/10, 1/100 and 1/1000 dilution of water samples. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Error bars represent standard error of the mean. Bars represent mean of three independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences where (***) $p < 0.001$. *E. coli* LPS was included as a positive control.

Similarly, “contaminated” bathing water samples induced TNF- α from MPI cells and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease ($p < 0.01$) in inducing TNF- α indicating that the main stimulant was attributable to LPS (Figure 5.9).

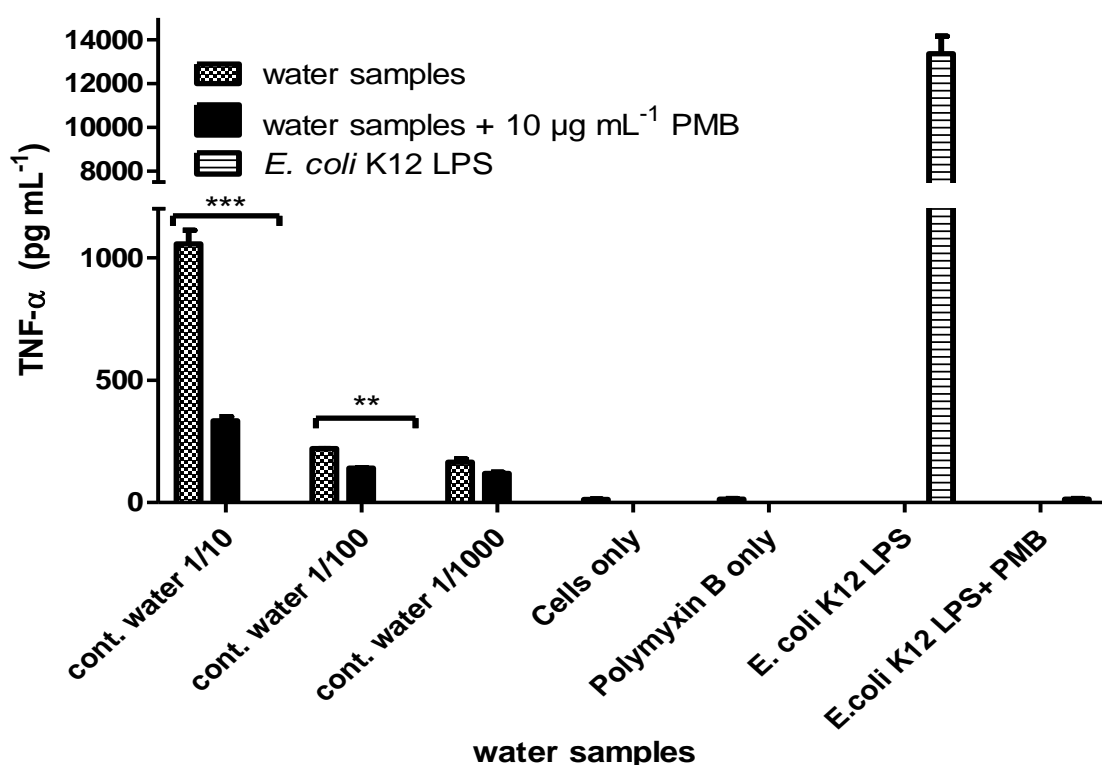


Figure 5.9: Effect of “contaminated” bathing water samples (cont. water, 135 EU mL⁻¹) on TNF- α cytokine secretion from the cell culture MPI measured using sandwich ELISA. Cells were treated with 1/10, 1/100 and 1/1000 dilution of water samples. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Error bars represent standard error of the mean. Bars represent mean of three independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences where (**) $p < 0.01$ and (***) $p < 0.001$. *E. coli* LPS was included as a positive control.

“S. bathe” water samples (slightly above the threshold of 50 EU mL⁻¹) induced TNF- α from MPI cells and showed a dilution effect. Similar water samples

treated with Polymyxin B showed a significant decrease ($p < 0.001$) in inducing TNF- α indicating LPS was the main stimulant (Figure 5.10).

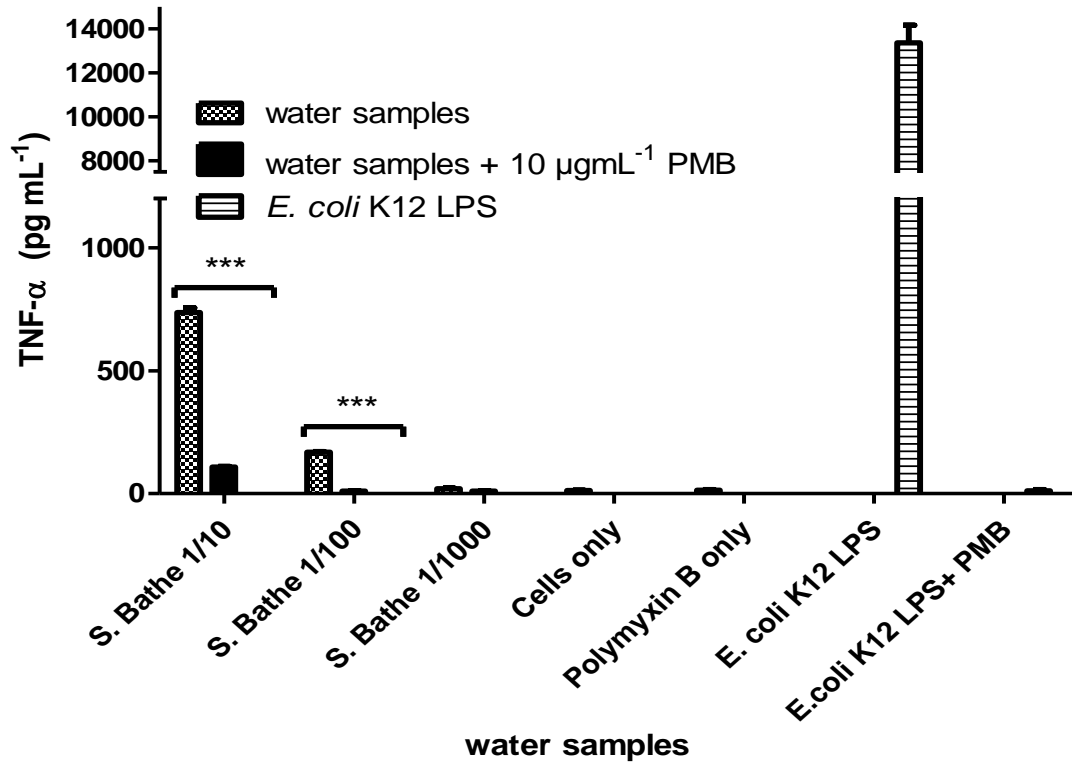


Figure 5.10: Effect of “S. bathe” (58 EU mL $^{-1}$) marine bathing water sample on TNF- α cytokine secretion from the cell culture MPI measured using sandwich ELISA. Cells were treated with 1/10, 1/100 and 1/1000 dilution of water samples. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Error bars represent standard error of the mean. Bars represent mean of three independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences. (***) $p < 0.001$. *E. coli* LPS was included as a positive control.

“Clean” marine bathing water samples induced TNF- α from MPI cells and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease ($p < 0.001$) in inducing TNF- α indicating LPS was the main stimulant (Figure 5.11).

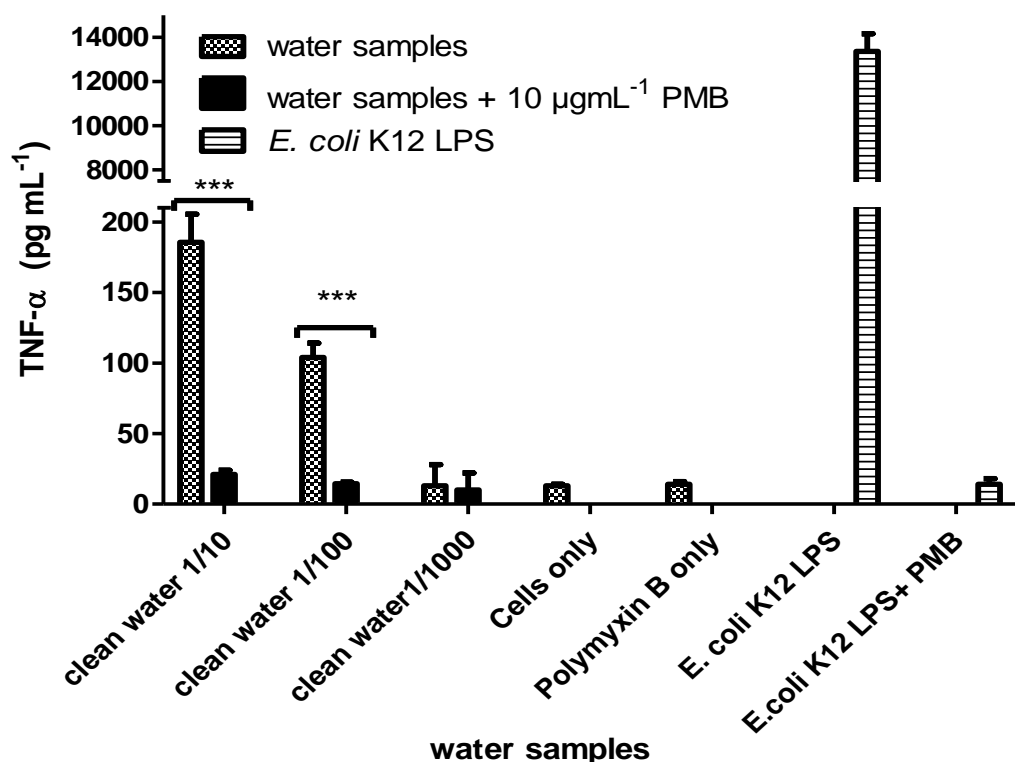


Figure 5.11: Effect of “clean” (6 EU mL⁻¹) marine bathing water sample on TNF- α cytokine secretion from the cell culture MPI measured using sandwich ELISA. Cells were treated with 1/10, 1/100 and 1/1000 dilution of water samples. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Error bars represent standard error of the mean. Bars represent results of three independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences. (***) $p < 0.001$. *E. coli* LPS was included as a positive control.

Results clearly show that the main stimulant in marine bathing water samples was due to the presence of LPS and the secretion of TNF- α showed a dilution effect. In order to show the significant differences between water samples in the stimulation of MPI cells and secretion of TNF- α , the levels of induced TNF- α from the least dilution (1/10 of each water sample) with PBM were subtracted from the least dilution (1/10 of each water sample) total IL-1 α levels leaving only the effect of LPS. These levels were then converted into number of fold difference to the resting untreated cells (cells only) (Figure 5.12 B). In addition,

the total fold increase of TNF- α without subtracting the non-LPS is also shown in figure 5.12 A.

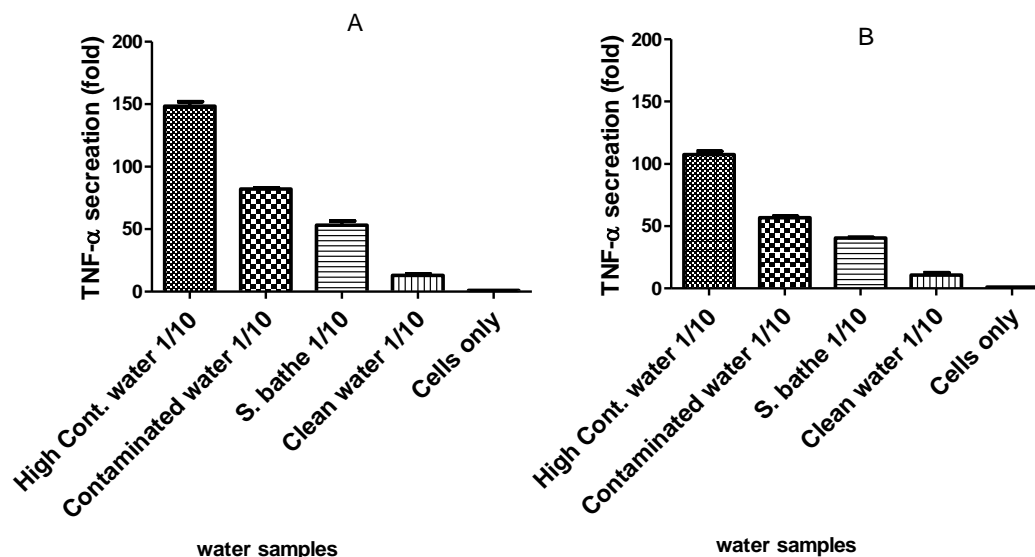


Figure 5.12: Effect of bathing water samples (High cont. water 230 EU mL⁻¹, “S. bathe” 58 EU mL⁻¹ and “clean” water 6 EU mL⁻¹) in the secretion of TNF- α . Results are expressed as the number of fold increase compared to cells only (A) represent the total fold increase of TNF- α (LPS and non LPS stimulants) (B) fold increase after deducting the levels of non-LPS stimulants by taking the difference between total and TNF- α and TNF- α release from water samples with PMB. One way ANOVA and Tukey’s HSD was carried out to determine significant differences. All showed a significant difference of $p < 0.01$, except between High cont. the rest where $p < 0.001$.

From the results above, there was a clear evidence that “highly contaminated” bathing water induced the highest secretion of TNF- α in comparison to “clean” bathing waters and that was mainly attributed to the LPS. Furthermore, the total secretion levels of TNF- α induced by LPS and non-LPS stimulants in “highly contaminated” water samples were significantly higher than “clean” bathing water.

5.5.1.3 IL-6 secretion

The levels of IL-6 induced by water samples from the MPI cells were assayed. Results showed PMB at a concentration of $10 \mu\text{g mL}^{-1}$ were appropriate to abolish the effect of *E. coli* K12 LPS and the levels of induced cytokines were similar to the levels of untreated cells. Cytokine induction showed no significant difference between unstimulated cells and cells stimulated with Instant Ocean (Underworld, UK), signifying that salinity has no effect on cell line stimulation.

“Highly contaminated” bathing water samples induced IL-6 from MPI cells and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease ($p < 0.05$) in the induction of IL-6 indicating LPS was the main stimulant (Figure 5.13).

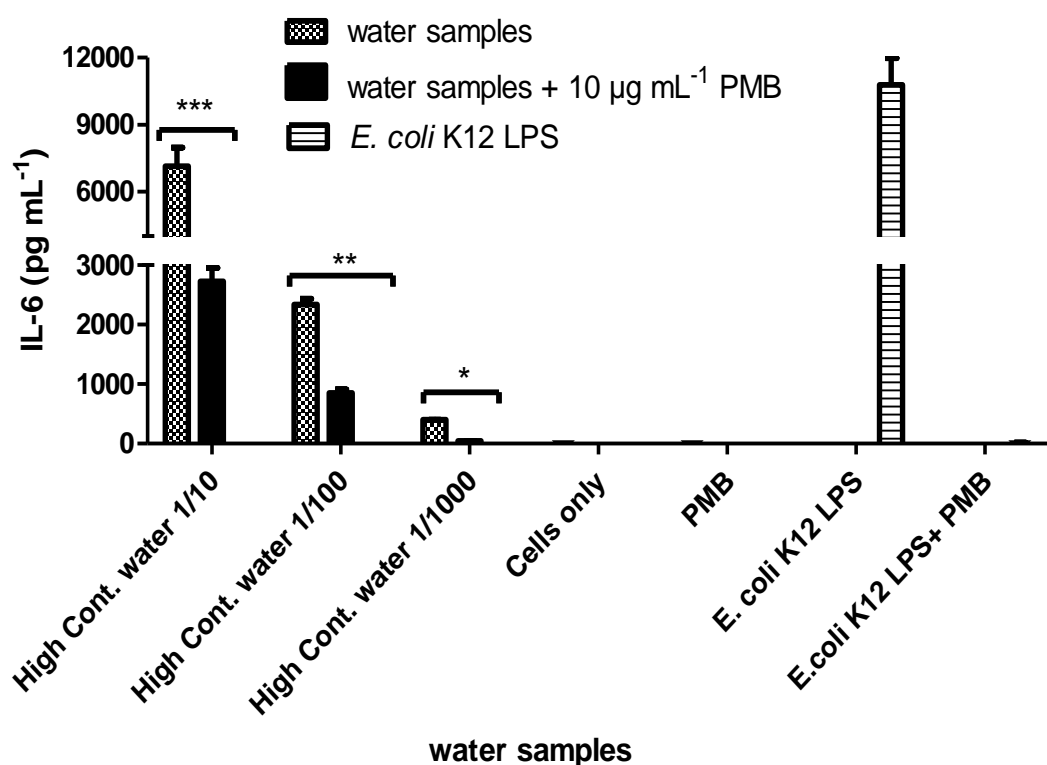


Figure 5.13: Effect of “Highly contaminated” marine bathing water samples (High cont. water, 230 EU mL⁻¹) on IL-6 cytokine secretion from the cell culture MPI measured using sandwich ELISA. Cells were treated with 1/10, 1/100 and 1/1000 dilution of water samples. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Error bars represent standard error the mean. Bars represent the mean of three independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was performed to determine significant differences. (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$. *E. coli* LPS was included as a positive control.

Likewise, “contaminated” marine bathing water samples (Cont. water, 135 EU mL⁻¹) induced IL-6 from MPI cells and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease ($p < 0.05$) in inducing IL-6 indicating that the main stimulant was attributable to LPS (Figure 5.14).

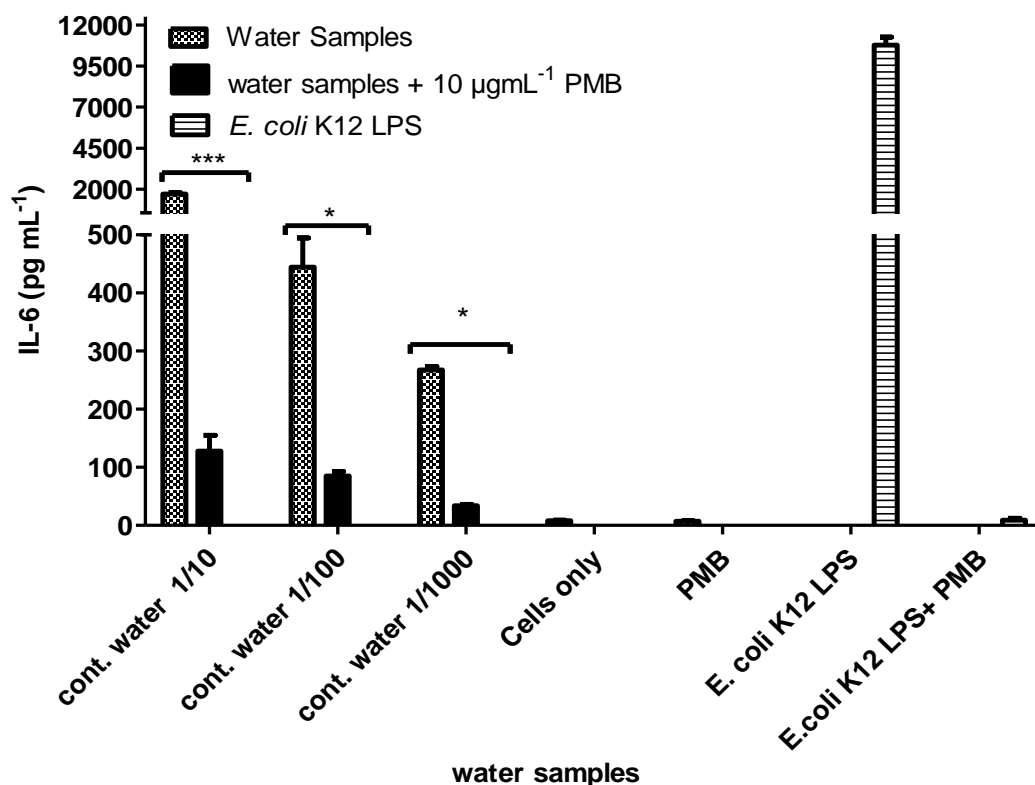


Figure 5.14: Effect of “contaminated” marine bathing water samples (Cont. water, 135 EU mL⁻¹) on IL-6 cytokine secretion in the cell culture MPI measured using sandwich ELISA. Cells were treated with 1/10, 1/100 and 1/1000 dilution of water samples. Polymyxin B (PMB) was added to similar water sample dilutions and used to stimulate the cells. Bars represent the mean of three independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was performed to determine significant differences. (*) $p < 0.05$, (***) $p < 0.001$. *E. coli* LPS was included as a positive control.

“S. bathe” water samples (slightly above the threshold of 50 EU mL⁻¹) induced IL-6 from MPI cells and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease ($p < 0.01$) in inducing IL-6 indicating LPS was the main stimulant (Figure 5.15).

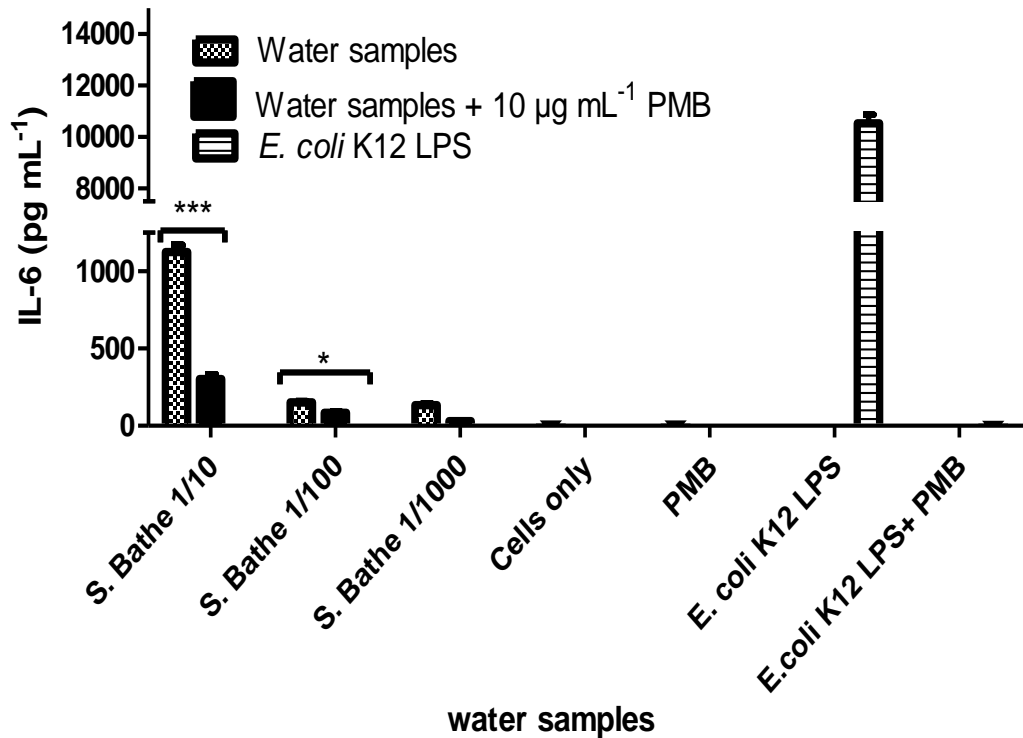


Figure 5.15: Effect of “S. bathe” (58 EU mL⁻¹) marine bathing water samples on IL-6 cytokine secretion from the cell culture MPI measured using ELISA. Cells were treated with 1/10, 1/100 and 1/1000 dilution of water samples. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Bars represent the mean of 3 independent experiments. Error bars represent standard error of the mean. One way analysis of variance (ANOVA) and Tukey’s HSD was performed to determine significant differences. (**) $p < 0.01$ and (***) $p < 0.001$. *E. coli* LPS was included as a positive control.

“Clean” marine bathing water samples induced IL-6 from MPI cells and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease ($p < 0.01$) in inducing IL-6 indicating LPS was the main stimulant (Figure 5.16).

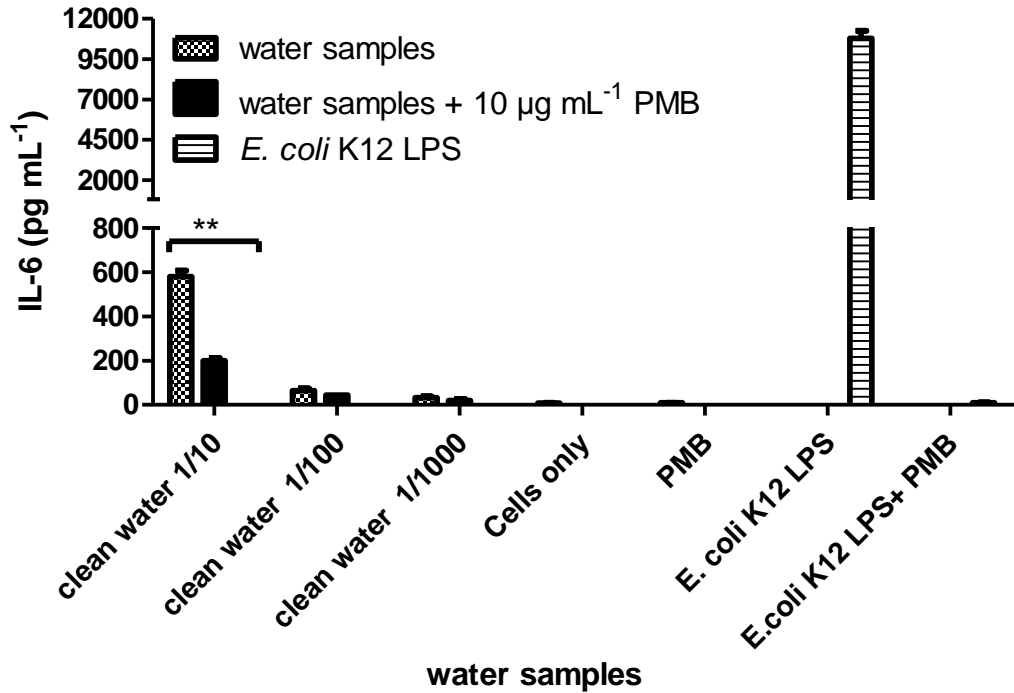


Figure 5.16: Effect of “clean” (6 EU mL⁻¹) marine bathing water samples on IL-6 cytokine secretion from the cell culture MPI measured using ELISA. Cells were treated with 1/10, 1/100 and 1/1000 dilution of water samples. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Error bars represent standard error of the mean. Bars represent the mean of three independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences. (**) $p < 0.01$. *E. coli* LPS was included as a positive control.

Results clearly show that the main stimulant in marine bathing water samples was due to the presence of LPS and the secretion of IL-6 showed a dilution effect. In order to show the significant differences between water samples in the stimulation of MPI cells and secretion of IL-6, the levels of induced IL-6 from the least dilution (1/10 of each water sample) with PBM were subtracted from the least dilution (1/10 of each water sample) total IL-6 levels leaving only the effect of LPS. These levels were then converted into the number of fold difference to the resting untreated cells (Figure 5.17 B). In addition the total fold increase of IL-1 α without subtracting the non-LPS is also shown in figure 5.17 A.

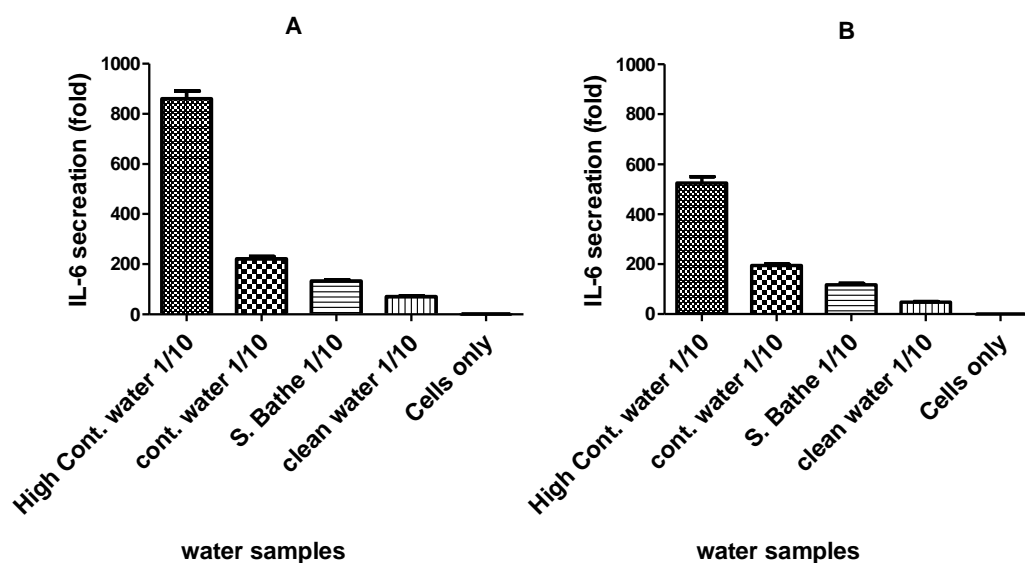


Figure 5.17: Effect of bathing water samples (High cont. water 230 EU mL⁻¹, “S. bathe” 58 EU mL⁻¹ and “clean” water 6 EU mL⁻¹) on the secretion of IL-6. Results are expressed as the number of fold increase compared to the control cells (A) represent the total fold increase of IL-6 (LPS and non LPS stimulants) (B) fold increase attributed to LPS only after deducting the levels of non-LPS stimulants. One way ANOVA and Tukey’s HSD was carried out to determine significant differences. All showed a significant difference of $p < 0.05$ except between “High contaminated” water and there rest where $p < 0.001$.

It is clearly showing that “highly contaminated” bathing water induced the highest secretion of IL-6 in comparison to “clean” bathing waters and that was mainly attributed to the LPS.

5.5.2 Results of stimulating the MM6 monocyte cell line

The levels of induced cytokines by water samples from the MM6 cells were assayed. Results indicated that PMB at a concentration of 10 µg mL⁻¹ were appropriate to inhibit the effect of *E. coli* K12 LPS and the levels of induced cytokines were similar to the levels of untreated cells. Cytokine induction showed no significant difference between unstimulated cells and cells stimulated with Instant Ocean (Underworld, UK), signifying that salinity has no

effect on cell line stimulation. 1/1000 water samples were initially investigated as stimulants showing that there was no significant difference in the induction of cytokines with untreated cells, hence, they were excluded from this experiment.

5.5.2.1 TNF- α secretion

“Highly contaminated”, “S. bathe” and “clean” bathing water samples induced TNF- α from MPI cells and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease ($p < 0.05$) in inducing TNF- α indicating LPS was the main stimulant (Figure 5.18).

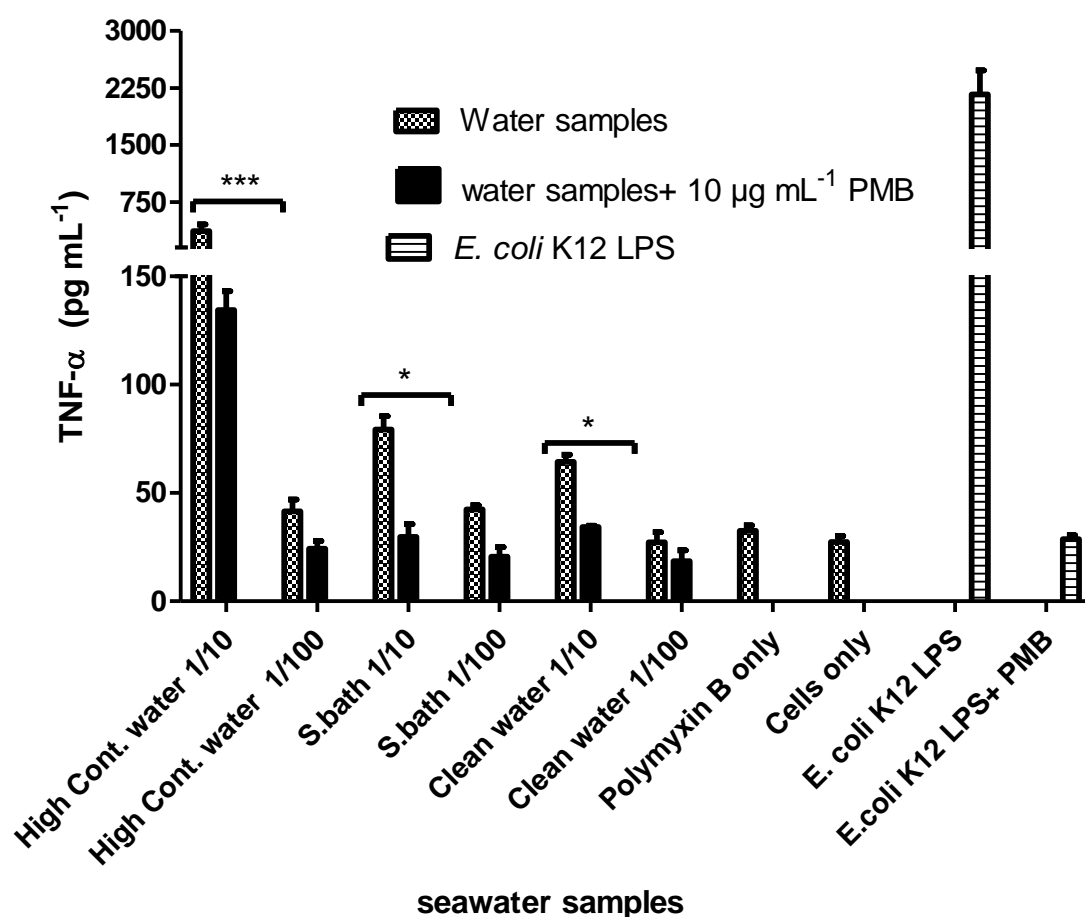


Figure 5.18: Effect of marine bathing water samples (High cont. water 230 EU mL⁻¹, “S. bathe” 58 EU mL⁻¹ and “clean” water 6 EU mL⁻¹) on TNF-α cytokine secretion from the cell culture MM6 measured using Luminex® xMAP multiplexing technology. Cells were treated with 1/10 and 1/100 dilutions of water samples. Polymyxin B (PMB) was added to similar water sample dilutions and used to stimulate the cells. Error bars represent standard error of the mean of three independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences. (*) $p < 0.05$, and (***) $p < 0.001$. *E. coli* LPS was included as a positive control.

Results evidently show that the main stimulant in marine bathing water samples was due to the presence of LPS and the secretion of TNF-α showed a dilution effect. In order to show the significant differences between water samples in the stimulation of MM6 cell line and secretion of TNF-α, the levels of induced TNF-α from the least dilution (1/10 of each water sample) with PBM were subtracted from the least dilution (1/10 of each water sample) total TNF-α levels leaving

only the effect of LPS. These levels were then converted into number of fold difference to the resting untreated cells (Figure 5.19 B). In addition the total fold increase of TNF- α without subtracting the non-LPS stimulant is also shown in figure 5.19 A.

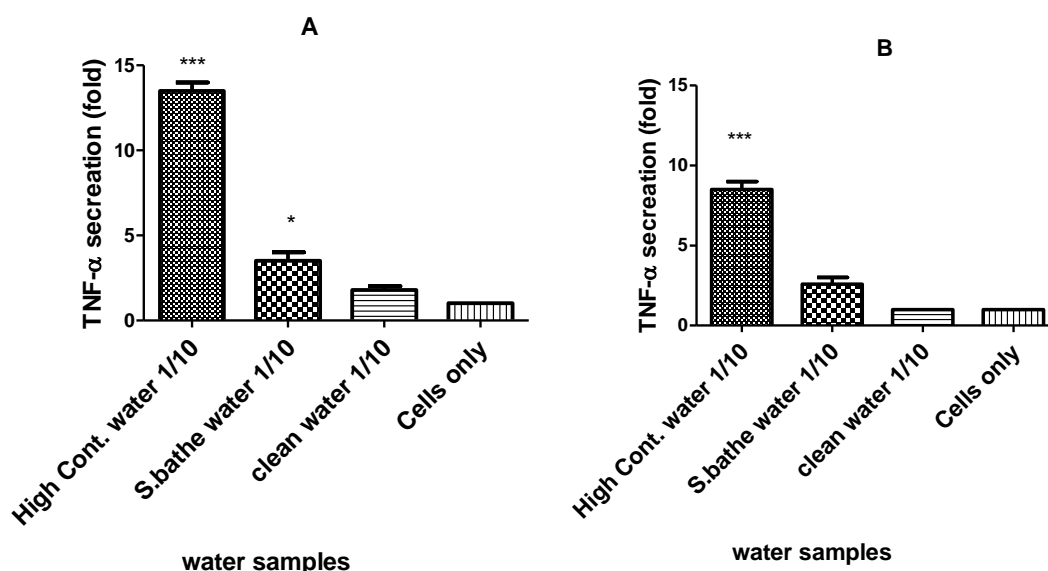


Figure 5.19: Effect of bathing water samples (High cont. water 230 EU mL⁻¹, “S. bathe” 58 EU mL⁻¹ and “clean” water 6 EU mL⁻¹) in the secretion of TNF- α from MM6 cell line. Results are expressed as a number of folds increase compared to the control cells (A) represent the total fold increase of TNF- α (LPS and non LPS stimulants) (B) fold increase attributed to LPS only after deducting the levels of non-LPS stimulants. Statistical analysis conducted using one way ANOVA and Tukey’s HSD was carried out to determine significant differences. High cont. water showed a significant difference of (***) $p < 0.001$ versus all other water samples, and “S. bathe” water in graph A showed a significant difference (*) $p < 0.05$ versus cells only.

It is apparent that “highly contaminated” bathing water samples induced the highest secretion of TNF- α in comparison to “clean” bathing waters and that was mainly attributed to the LPS.

5.5.2.2 IL-8 secretion

Results show that “highly contaminated” and “S. bathe” marine water samples induced IL-8 from MM6 cell line and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease in inducing IL-8 indicating LPS was the main stimulant. “Clean” bathing water samples induced no significant difference of IL-8 in comparison the untreated cells only as shown in figure 5.20.

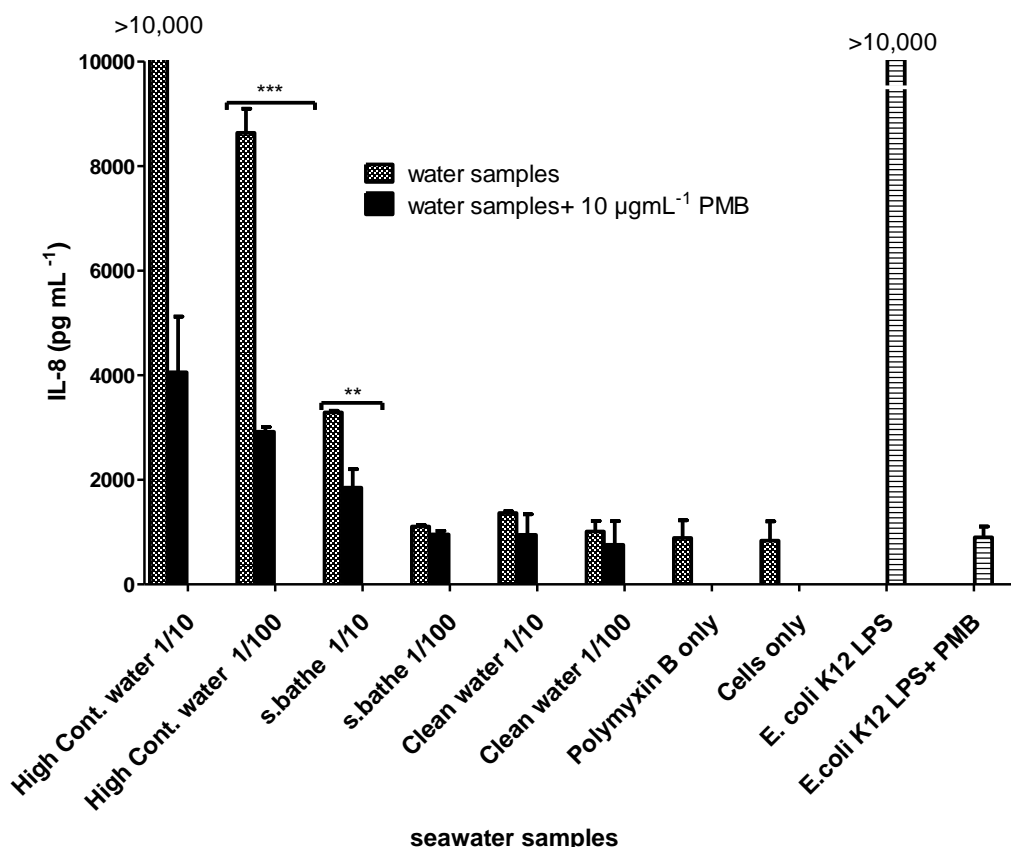


Figure 5.20: Effect of water samples (High cont. water 230 EU mL⁻¹, “S. bathe” 58 EU mL⁻¹ and “clean” water 6 EU mL⁻¹) on IL-8 cytokine secretion from the cell culture MM6 measured using Luminex[®] xMAP multiplexing technology. Cells were treated with 1/10 and 1/100 dilutions of water samples. Polymyxin B (PMB) was added to similar water samples dilutions and used to stimulate the cells. Error bars represent standard error of the mean. Bars represent the mean of three independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences. (**) $p < 0.01$ and (***) $p < 0.001$. *E. coli* LPS was included as a positive control.

Water sample with high LPS levels (1/10 “highly contaminated” water sample) induced IL-8 above the detection levels (above 10,000 pg mL⁻¹); the second dilution (1/100) was used for comparison in figure 5.21. Ideally, when levels of cytokines exceed the detection limit of an assay, supernatant would have been diluted and the assay would be repeated for that particular sample. However,

because running an additional assay is expensive, and because the second dilution (1/100) was within the detection range, repetition was unnecessary.

Results clearly show that the main stimulant in marine bathing water samples was due to the presence of LPS and the secretion of IL-8 showed a dilution effect.

In order to show the significant differences between water samples in the stimulation of MM6 cell line and secretion of IL-8, the levels of induced IL-8 from the dilution (1/100 of each water sample) with PBM were subtracted from the dilution (1/100 of each water sample) total IL-8 levels leaving only the effect of LPS. These levels were then converted into number of fold difference to the resting untreated cells (Figure 5.21 B). In addition the total fold increase of IL-8 without subtracting the non-LPS is also shown in figure 5.21 A.

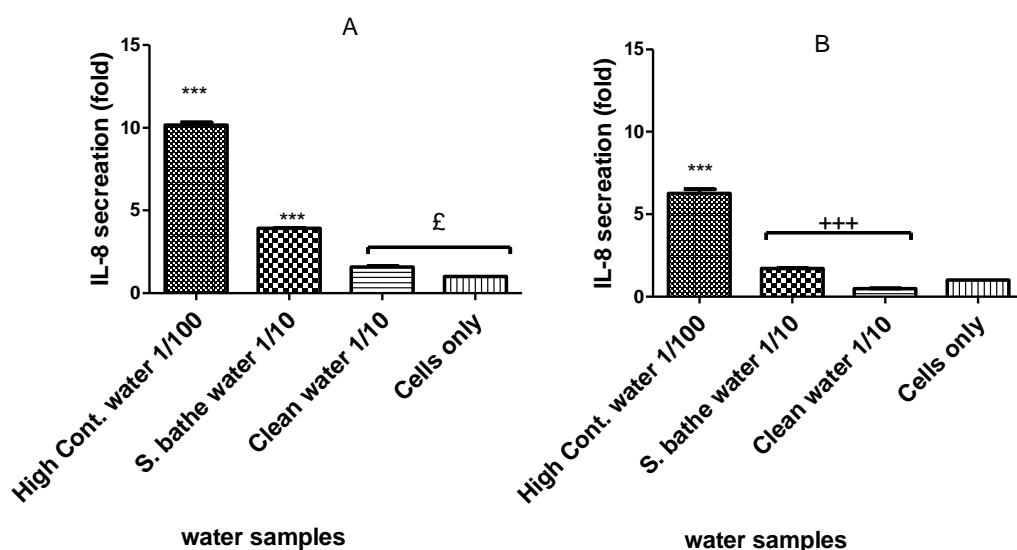


Figure 5.21: Effect of marine bathing water samples (High cont. water 230 EU mL⁻¹, “S. bathe” 58 EU mL⁻¹ and “clean” water 6 EU mL⁻¹) in the secretion of IL-8 from MM6. Results are expressed as the number of fold increase compared to the control cells (A) represent the total fold increase of IL-8 (LPS and non LPS stimulants) (B) fold increase attributed to LPS only after deducting the levels of non-LPS stimulants. One way ANOVA and Tukey’s HSD was carried out to determine significant differences. (***) $p < 0.001$ versus the rest of water samples, (£) $p < 0.05$ between “clean” water and cells only and (+++) $p < 0.001$ between “S. bathe” and “clean” water. *E. coli* LPS was included as a positive control.

It is clearly showing that “highly contaminated” bathing water induced the highest secretion of IL-8 in comparison to “clean” bathing waters and that was mainly attributed to the LPS.

5.5.2.3 IL-6 secretion

From the data in figure 5.22, it is apparent that the “highly contaminated”, “S. bathe”, and “clean” bathing water samples induced IL-6 from MM6 cell line and showed a dilution effect. Similar water samples treated with Polymyxin B showed significant decrease in inducing of IL-6 indicating LPS was the main stimulant.

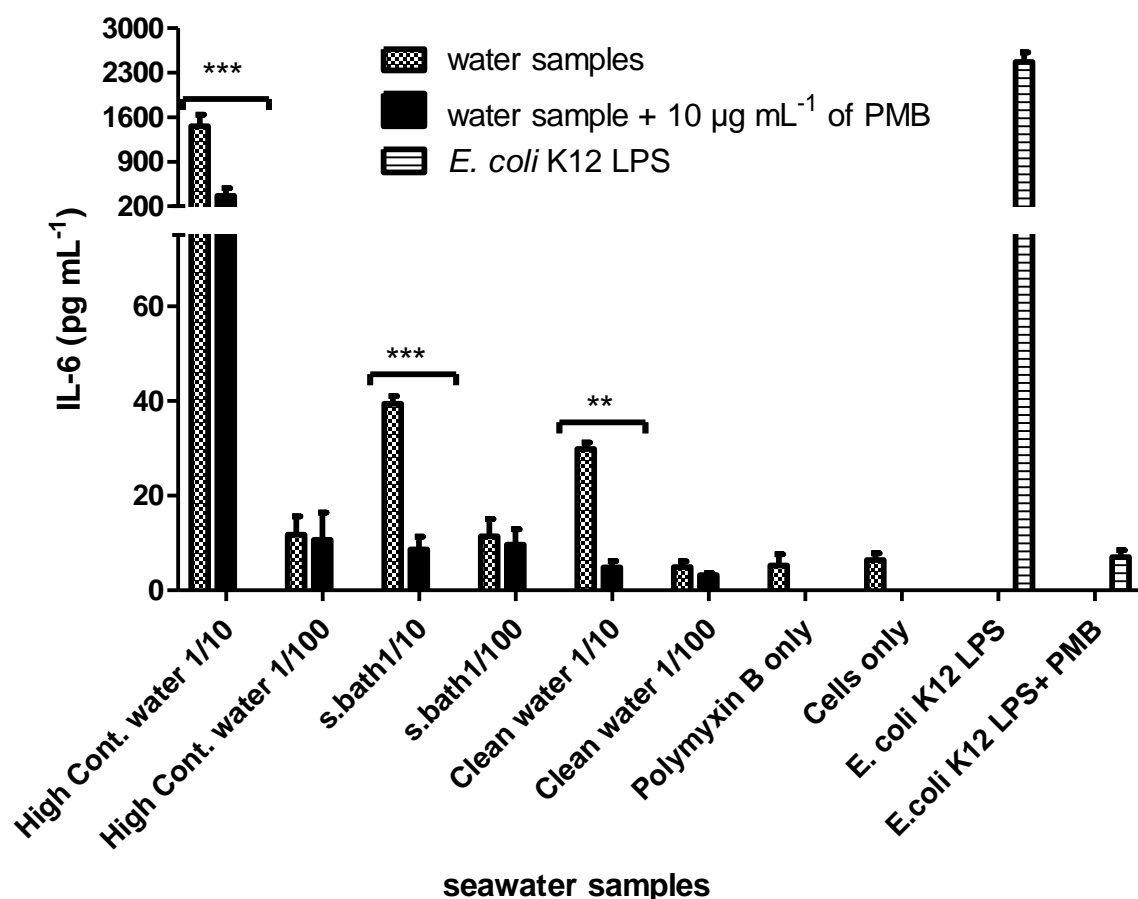


Figure 5.22: Effect of water samples (High cont. water 230 EU mL⁻¹, “S. bathe” 58 EU mL⁻¹ and “clean” water 6 EU mL⁻¹) on IL-6 cytokine secretion from the cell culture MM6 measured using Luminex® xMAP multiplexing technology. Cells were treated with 1/10 and 1/100 dilutions of water samples. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Error bars represent standard error of the mean. Bars represent the mean of three independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences. (**) means $p < 0.01$ and (***) $p < 0.001$. *E. coli* LPS was included as a positive control.

Results clearly show that the main stimulant in marine bathing water samples were due to the presence of LPS and the secretion of IL-6 showed a dilution effect. In order to show the significant differences between water samples in the stimulation of MM6 cell line and secretion of IL-6, the levels of induced IL-6 from the least dilution (1/10 of each water sample) with PBM were subtracted from the least dilution (1/10 of each water sample) total IL-6 levels leaving only the

effect of LPS. These levels were then converted into number of fold difference to the resting untreated cells (Figure 5.23 B). In addition the total fold increase of IL-6 without subtracting the non-LPS is also shown in figure 5.23 A.

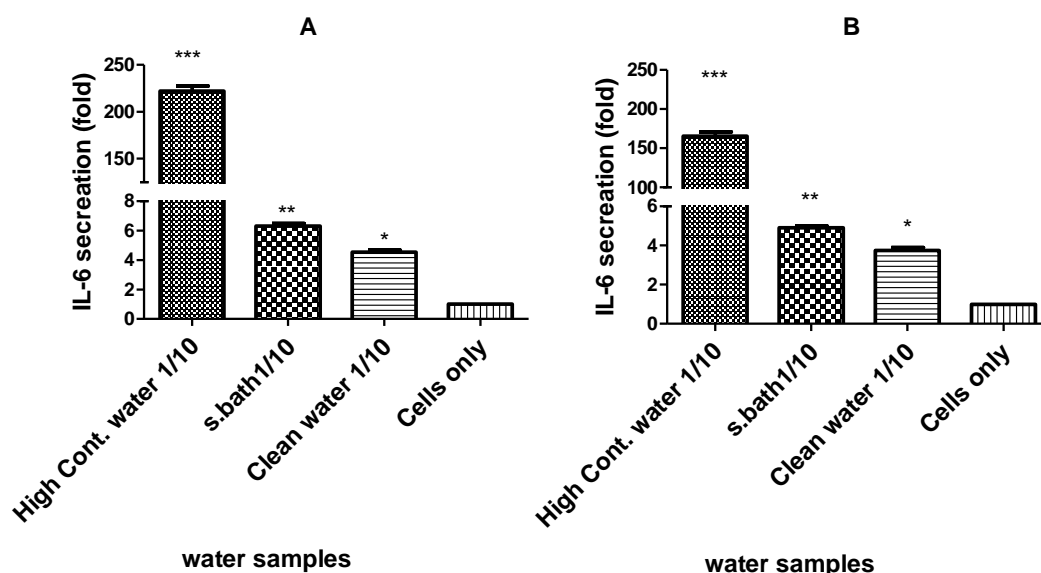


Figure 5.23: Effect water samples (High cont. water 230 EU mL⁻¹, "S. bathe" 58 EU mL⁻¹ and "clean" water 6 EU mL⁻¹) on the secretion of IL-6 from MM6. Results are expressed as the number of fold increase compared to the control cells (A) represent the total fold increase of IL-6 (LPS and non LPS stimulants) (B) fold increase after deducting the levels of non-LPS stimulants by taking the difference between total and TNF- α and IL-6 release from water samples with PMB. One way ANOVA and Tukey's HSD was carried out to determine significant differences. (***) $p < 0.001$ versus the rest of water samples, (**) $p < 0.01$ between "S. bathe" and cells only and (*) $p < 0.05$ between "clean" water and cells only.

It is clearly showing that "highly contaminated" bathing water induced the highest secretion of IL-6 in comparison to "clean" bathing waters and that was mainly attributed to the LPS. In addition, the total secretion levels of IL-6 induced by LPS and non-LPS stimulants in "highly contaminated" water samples were significantly higher than "clean" bathing water.

5.5.2.4 IL-1 α secretion

Results show that “highly contaminated” and “S. bathe” marine water samples induced IL-1 α from MM6 cell line and showed a dilution effect. Similar water samples treated with Polymyxin B showed a significant decrease in inducing IL-1 α indicating LPS was the main stimulant. “Clean” bathing water induced no significant difference of IL-1 α in comparison the untreated cells only as shown in figure 5.24.

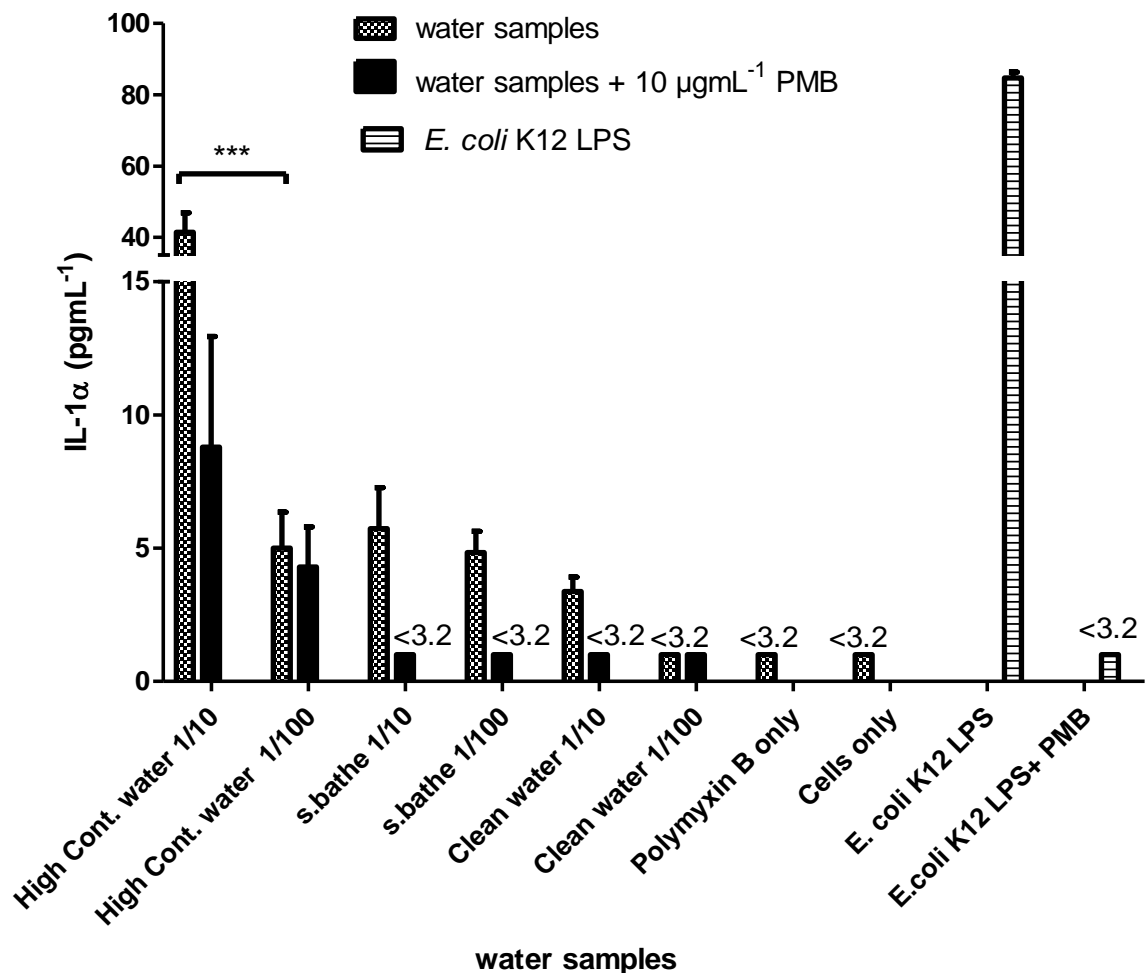


Figure 5.24: Effect of water samples (High cont. water 230 EU mL⁻¹, “S. bathe” 58 EU mL⁻¹ and “clean” water 6 EU mL⁻¹) on IL-1α cytokine secretion from the cell culture MM6 measured using Luminex® xMAP multiplexing technology. Cells were treated with 1/10 and 1/100 dilutions of water samples. Polymyxin B (PMB) was added to similar water samples dilution and used to stimulate the cells. Error bars represent standard error of the mean. Bars represent the mean of three independent experiments. One way analysis of variance (ANOVA) and Tukey’s HSD was carried out to determine significant differences. (***) $p < 0.001$. *E. coli* LPS was included as a positive control.

It appears that only “highly contaminated” bathing water sample was able to induce a significantly different level of IL-1α from the MM6 cell line. Since the lowest level of cytokine that can be detected is 3.2 pg mL⁻¹, statistical analysis cannot be performed on results below that level.

5.6 Discussion

Cell culture models have been used since their establishment early in the last century to screen for the health risks and toxicity of drugs, chemicals, etc. (Bradlaw, 1986). Previously conducted studies to assess the health effects of contaminated bathing waters were based on epidemiological surveys (Cabelli, 1983, Cabelli, 1989, Cabelli *et al.*, 1979, Cabelli *et al.*, 1982, Cabelli *et al.*, 1983, Kay *et al.*, 1994); and have no direct relevance to potential health risks, hence a more robust method is required. Therefore, the main aim of this chapter was to *in vitro* investigate the inflammatory effects of contaminated bathing waters. To the best of the author's knowledge, this is the first *in vitro* study that investigates their inflammatory effects. These water samples were used to stimulate two cell culture models *in vitro* to investigate the proinflammatory effects of LPS in "clean" and "contaminated" bathing water samples. The first cell culture model was mouse macrophage MPI cells which has many characteristics of alveolar macrophages (Fejer *et al.*, 2013). The second cell culture model was a human blood monocytic cell line MM6. Both cell culture models have shown previously to be good *in vitro* models to study LPS exposure (Liu *et al.*, 2011, Zughaier *et al.*, 1999a, Zughaier *et al.*, 1999b, Fejer *et al.*, 2013). Four cytokines (TNF- α , IL-1 α , IL-6 and IL-8) were investigated after stimulation of MM6 cell line and three cytokines (TNF- α , IL-1 α and IL-6) were investigated after stimulation of MPI cells with marine bathing water samples. Results showed that "highly contaminated" water sample (230 EU mL⁻¹) significantly induced higher levels of cytokines from both MM6 and MPI cells in comparison to "clean" bathing water (6 EU mL⁻¹). The use of PMB, which has the effect of neutralising LPS,

markedly abolished the induction of cytokines induced by *E. coli* K12 LPS confirming that the major inflammatory stimulant in marine water samples was attributed to LPS whereas PMB itself had no effect on cytokine production.

PMB is a cyclic cationic polypeptide antibiotic produced by *Paenibacillus polymixa*. PMB is commercially produced and widely used as LPS neutraliser by blocking the biological effects of LPS through binding to the negatively charged lipid A moiety (Palmer and Rifkind, 1974). The hydrophobic amino acids phenylalanine and leucine of Polymyxin B interact with the hydrophobic bonds of the fatty acid portion of the lipid A moiety of LPS. The negatively charged phosphate groups of the lipid A moiety binds to the amino groups of Polymyxin B via ionic bonds leading to a very strong interaction with an unlikelihood of dissociation (Zavascki *et al.*, 2007). Minor levels of cytokines induced from PMB-treated water samples can be attributed to non LPS stimulants that could be anything from cellular components of Gram-positive bacteria (Orman and English, 2000), chemicals (Joris *et al.*, 2013) , etc.

The salinity of seawater was excluded to be affecting the osmotic pressure of cells based on the results obtained by cells stimulation with simulated seawater only which showed to have no significant difference in cytokine induction with unstimulated cells.

Several studies have documented that LPS can cause various health risks. Health risks due to LPS present in contaminated water could cause respiratory, digestive, skin, and ocular health risks (Stewart *et al.*, 2006). A study conducted by Sandstorm *et al* (1992) has shown that inhalation of LPS could cause

chronic lung function impairment. Hultin *et al* (2011) conducted a study that showed LPS could be aerosolised by wave action in marine waters, hence increasing the chance of LPS inhalation by surfers and swimmers. Another study conducted by Coye *et al* (1989) suggested LPS could cause asthma-like symptoms when inhaled. In human, acute inhalation of LPS can lead to various lung-related symptoms such as dry cough, decrease in lung function, fever, headache and sometimes dyspnoea and lung injury (DECOS, 1998, Kao *et al.*, 2007). These symptoms could develop within 4 hours of exposure to LPS and could be worse in humans with asthma or inflammation of the nasal mucosa. It has been documented that LPS can play a synergistic effect and can amplify the effect of allergen and induce high levels of cytokines from stimulated macrophages (Michel, 2000).

Results here suggest that MPI lung-like macrophages were a good model cells in this study to investigate the proinflammatory effects of contaminated bathing water samples. The use of MPI cells in this study shed light on potential human health risks related to lung since LPS was the main stimulant.

Kay *et al.* (1994) gastroenteritis could take place even when bathing waters pass the European Union directive testing. Patois and colleagues (2000) suggested that gastroenteritis could take place when water containing LPS from cyanobacteria is ingested which aggress with Keleti *et al.* (1979). Also it is possible for LPS to migrate to the blood stream as suggested by Kiefer and Ali-Akbarian (2004). In the human body, Once LPS reaches the blood stream; the classical LPS recognition pathway by key immune cells (Iwasaki and Medzhitov, 2010) culminating by proinflammatory reaction.

Here in this study, a human blood monocyte MM6 cell line was used as a model to shed light on the potential biological relevance of LPS present in contaminated marine bathing water samples. The use of MM6 and MPI cell culture models may contribute to the assessment of health impacts of contaminated bathing water. Other studies correlated LPS in marine bathing waters with ocular (Schultz *et al.*, 2000) and skin (Pitois *et al.*, 2000) health risks.

At present, the only reliable method to prevent LPS-related health risks and thus diseases is by limiting exposure. However, shedding light on early biological responses to LPS exposures may well facilitate the development of schemes for early recognition and disease prevention. For instance, increased serum levels of TNF- α and IL-8 have been reported in workers occupationally exposed to nylon flock used to make microfiber garments (Atis *et al.*, 2005). While the exact role of produced cytokines in toxicity induced by nylon flock is unknown, precautionary measures have already been implemented. For LPS exposure, the development and establishment of effective methods to monitor for such exposure by testing marine bathing water for LPS levels might have a useful method to decrease health risks and preventing of disease occurrence. While it is almost impossible to eliminate the exposure to LPS, especially in LPS-rich environment such as seawater, it is essential to set a “safe” LPS exposure levels.

We have sought in this study to assess the threshold at which health problems might arise from being in contact with high LPS levels. Results suggest that if 50 EU mL⁻¹ is a reasonable threshold that might be considered as a cut off value for safe bathing (Chapter 3), which is reflected in the production of

increased cytokine levels. Raising awareness to the increase in LPS levels could be an effective method in the prevention of bathing water-related health risks. A standard safe LPS level and determination method should be implemented in the next European Union water directive.

In conclusion, two cell culture models have been used to explore the potential biological relevance of LPS present in contaminated marine bathing water samples. Results showed that increased levels of LPS present in marine bathing water samples were associated with induction of high levels of proinflammatory cytokines. These cell culture models mimic murine lung-like macrophages and human blood monocytes. Results here suggest that these *in vitro* inflammatory cell models may contribute to the assessment of health impacts of LPS exposure from marine bathing water samples. However, more research on this topic needs to be undertaken before the association between LPS levels and assessment of health impacts of LPS exposure is more clearly understood. In future investigations, it might be possible to use human skin, intestine and ocular cell culture lines as well as animal models which will deepen our understanding regarding health risks from exposure to contaminated bathing waters.

Chapter 6: General discussion and conclusion

The quality of marine bathing waters is currently monitored using traditional culture-based methods; however, a major problem with this kind of application amongst several drawbacks is retrospective results. In recent years, there has been an increasing interest in rapid methods to assess the bacteriological quality of marine bathing waters to be used by bathers and beach goers. However, it is very difficult to find a single universal biomarker to monitor the biological status of marine bathing waters. Results of this research have successfully fulfilled the objectives identified in the rationale. To summarise, this research has:

- A. Successfully optimised a Kinetic QCL™ LAL assay to measure total LPS and overcome salinity interference in marine bathing water.
- B. Successfully isolated and enumerated the levels of FIB in addition to *Bacteroides* species and total coliforms over a summer period (2012, Chalfont beach, UK).
- C. Successfully correlated the number of bacterial indicator CFU from bathing seawater to the total LPS in these waters, which can be used as a rapid biomarker for bacterial contamination in such waters using the quantitative Kinetic QCL™ LAL assay.
- D. Recommended a cut off value of 50 EU mL⁻¹ for LPS threshold for bathing suitability.
- E. Successfully designed and tested a portable, rapid (approximately 20 minutes), endpoint, semi-qualitative (pass/fail) LAL assay to investigate the bacteriological quality of marine bathing water by measuring the levels of total LPS.

- F. Successfully used *Bacteroides* host specific molecular biomarker to source track the faecal pollution.
- G. Successfully isolated and speciated five different marine environmental *Bacteroides* species.
- H. Successfully profiled extracted LPS from five *Bacteroides* species for LPS banding pattern similarity using SDS-PAGE.
- I. Successfully investigated the biological activity of LPS from five *Bacteroides* species in both cell culture models and LAL assay.
- J. Successfully investigated the inflammatory effects of “clean” and “contaminated” bathing waters using *in vitro* cell culture models by monitoring the levels of proinflammatory cytokines.

Water samples were collected from Challaborough beach and levels of FIB were monitored using traditional culture based methods. A sensitive Kinetic QCL™ LAL assay was successfully optimised to monitor the levels of total LPS in marine bathing waters. The optimisation included overcoming the salt interference with the assay by diluting water samples 1:100 fold, which also scaled the LPS levels to a detectable level when using the endpoint LAL assay. Total LPS highly correlated with the numbers of Gram-negative faecal indicator bacteria *E. coli* and also *Bacteroides* species showing an increase whenever there was an increase in the latter mentioned bacteria and the levels decline when low bacteria numbers were recorded. The levels of LPS also seemed to decline from the bathing water within the time period of the next sample (1 week), possibly within three days as suggested by Shibata *et al.* (2009).

In addition, this established and suggested a cut off value of 50 EU mL⁻¹, above which seawater considered unsuitable for bathing. In this research, a rapid method has been developed to test the quality of marine bathing waters by targeting total LPS as a biomarker.

The background level of total LPS in “excellent” bathing water samples was shown to be 6 EU mL⁻¹ and total LPS levels increase with the increase of FIB. The Kinetic QCL™ LAL assay is considered to be extremely rapid, taking only 23 minutes to perform and requires no extraction or pre-processing except for a simple dilution step. Although this method, to the best knowledge of the author, is by far the fastest method that can be conducted providing quantitative results, it was still not portable and requires sophisticated equipment and specialised software. Since the main idea was to develop a method that can be conducted by untrained personnel such as beach goers, a simplified qualitative, semi-quantitative, endpoint LAL assay was developed and optimised to assess the bacteriological activity of marine bathing waters also targeting total LPS and taking approximately 20 minutes. An endpoint LAL assay was successfully optimised and tested in real time on the study area (Challaborough beach). Various parts of the beach were tested and results were comparable with the number of FIB; the colour intensity of the end product of the LAL assay corresponds reciprocally with the level of enumerated FIB. Light purple colour development (bellow 50 EU mL⁻¹) when low levels of FIB were enumerated and dark purple colour when high levels of FIB were recorded. A cut off of LPS standard was used simultaneously for comparison. This method is currently being developed and potentially commercialised in the near future. This method

is not an attempt to supersede current culture based methods; it is simply a rapid method to assess the quality of marine bathing water without the need to wait for at least 24 hours. Molecular qPCR methods have been previously developed that are able to rather rapidly monitor the levels of FIB within approximately four hours; however, qPCR methods are expensive, require pre-processing and extraction methods in addition to an equipped laboratory with qualified technician. qPCR method is still slower than our LAL assay and cannot be performed on site (Chern *et al.*, 2009, Miller, 2013).

Although in this research total LPS levels were shown to be associated with faecal bacteria (*E. coli* and *Bacteroides*), it is possible that high levels of total LPS might be observed in non-faecal associated bacterial contamination. Despite the fact that total LPS levels could rise because of various Gram-negative bacterial increase such as cyanobacteria blooms or an increase in *Vibrio* densities for example, the immunological assay showed that increasing in the levels of total LPS can potentially cause human health problems (Chapter 5). Hence, even when levels of total LPS drastically increase in a non-FIB contamination, it would still be risky to bathe in such waters based on results from chapter five. However, a more faecal-specific biomarker was thought to be more appropriate to overcome the increase of non-faecal bacteria associated LPS. Therefore, the LPS of *Bacteroides* species was chosen as a specific faecal biomarker. *Bacteroides* isolated from marine bathing water samples were speciated using phenotypic and genotypic methods showing five distinctive *Bacteroides* species.

Systematic further investigation including LPS profiling of *Bacteroides*, biological activity of extracted LPS of *Bacteroides* was conducted. The potential biological relevance of extracted *Bacteroides* species LPS was also investigated in a human monocytic cell line (MM6) and mouse alveolar-like macrophage (MPI) by measuring the levels of induced proinflammatory cytokines.

LPS profiling of five *Bacteroides* species using SDS-PAGE showed high similarity in banding pattern indicating similarity in their structures. Both LAL and immunological activity assays showed that *Bacteroides* LPS of all the five species isolated was approximately 300 and 100 fold less potent than *E. coli* reference LPS respectively. The finding of the biological activity of *Bacteroides* LPS was consistent with those of Rietschel *et al* (1994) who found that the LPS of *Bacteroides fragilis* was 100-1000 fold less potent than *E. coli* LPS. This indicates that the LPS of *Bacteroides* induce low levels of cytokines. In addition, one of the preferable criteria of a biomarker indicator for faecal contamination is that it would be non-pathogenic; this is another advantage in using *Bacteroides* LPS for this purpose. In order to selectively detect LPS of *Bacteroides* species to be used as a rapid method for assessing the quality of marine bathing waters an ELISA assay was designed using commercially available antibodies. Different ELISA setups and troubleshooting protocols were attempted to assess the problem with the anti-*Bacteroides* antibody, however, after spending time endeavouring to optimise the assay, it seems that the antibodies were faulty and unfunctional. The anti-*Bacteroides* antibody supplier was contacted to provide feedback and information regarding the anti-*Bacteroides* antibody,

however, no useful answers were provided. It has been thought of raising antibodies against extracted LPS of *Bacteroides* species, however, with the time constraints and costs; this has been suggested as future work. Overall, results suggest that LPS of *Bacteroides* species have a high potential and can be developed as a rapid method using either ELISA or preferably lateral flow immunoassay.

Lack of systematic methods to investigate the health of humans in relation to contaminated marine bathing waters with bacterial and total LPS has resulted in a gap in knowledge. In the United States of America alone, thousands of people develop illness related to recreational waters (Sinclair *et al.*, 2009). In the literature, the only methods used to assess the effect of contaminated bathing waters on human health is by surveying beach goers and answering questions a few days after going to the beach. Since such method is unable to differentiate whether illness occurred because of actually bathing or non-bathing related reasons; a different method is preferable to assess the biological relevance of contaminated bathing water to human health. Part of the aim of this project is to develop a method to evaluate inflammatory effects of “contaminated” and “clean” bathing waters using cell culture models. The inflammatory effects of these water samples were investigated by measuring the induction of proinflammatory cytokines after stimulating human monocytic (MM6) and mouse alveolar-like macrophage (MPI) cells. Results showed that there was a significant difference in the induction of proinflammatory cytokines between “clean” and “contaminated” bathing waters. In the presence of LPS inhibitor (PMB), the production of proinflammatory cytokines significantly

decreased indicating that the LPS present in bathing water samples was the major stimulant. Results also suggested that MPI and MM6 cells showed to be good *in vitro* models in shedding light on potential inflammatory effects of LPS exposure from contaminated bathing water.

Results of this study also highlighted the gap that should be covered by the European Union directive of bathing waters since there are no regulations concerning levels of total LPS. Several studies have explored the synergistic effects of LPS with different diseases (Bodet and Grenier, 2010, Worthen *et al.*, 1987, Drost *et al.*, 1999, Wollin *et al.*, 2001, Davidson *et al.*, 2002, SchÜTte *et al.*, 1997, Yamada *et al.*, 2000, Yanagisawa *et al.*, 2003, Sikka *et al.*, 2001, Nakajima *et al.*, 1987) especially pulmonary diseases. Other studies have shown that bacteria and LPS can be aerosolised by wave action (Coye and Goldoft, 1989) in seawater and such aerosol inhaled in addition to the ingestion by bathers could lead to health problems. Hence, it is essential to investigate the levels of total LPS in marine bathing waters using the optimised LAL assay (Chapter 3) to ensure human health and avoid unnecessary health complications. Hence, it was thought that this LAL assay can be further developed (undergoing) to be used by untrained beach goers and untrained water-sports performers.

Possible drawbacks identified in using the LAL assay was that the shelf life of reagents; once the reagents prepared, they need to be used within one week. Furthermore, the possible drawback of a false positive event (although never occurring in this study) when levels of total LPS could increase higher than the suggested threshold (50 EU mL⁻¹) has consequences. This increase in total

LPS maybe not attributable to faecal contamination, and this would give the impression of false evaluation of the quality of marine bathing waters. However, such an increase in total LPS could potentially cause human health risks as shown in results presented in chapter five.

Further research should be done to:

- I. Simplify and refine our developed LAL assay to easily test the quality of marine bathing waters (undergoing).
- II. Identify the total LPS background levels in different beaches and correlate it to the levels of FIB.
- III. Raise antibodies against purified *Bacteroides* LPS and develop an ELISA assay technique using methods described in chapter four and further develop a more faecal-specific biomarker in marine bathing waters.

In conclusion, the significance of this study is a rapid method has been developed to test the quality of marine bathing waters using a commercially available LPS detection kit. *Bacteroides* LPS warrants a promising faecal-specific biomarkers and could be developed and used as a rapid faecal-indicator. The inflammatory effects of contaminated bathing waters on *in vitro* cell culture models showed an increase in the induction of proinflammatory cytokines which was mainly attributed to total LPS. It is suggested that the next European Union directive consider legislation of monitoring the levels of total LPS in marine bathing waters since it could have adverse health effects on human health.

References

- AHMED, W., HUYGENS, F., GOONETILLEKE, A. & GARDNER, T. 2008. Real-Time PCR Detection of Pathogenic Microorganisms in Roof-Harvested Rainwater in Southeast Queensland, Australia. *Applied and environmental microbiology*, 74, 5490-5496.
- ALEXANDER, C. & RIETSCHER, E. T. 2001. Invited review: Bacterial lipopolysaccharides and innate immunity. *Journal of Endotoxin Research*, 7, 167-202.
- ALHAWI, M., STEWART, J., ERRIDGE, C., PATRICK, S. & POXTON, I. R. 2009. *Bacteroides fragilis* signals through Toll-like receptor (TLR) 2 and not through TLR4. *Journal of Medical Microbiology*, 58, 1015-1022.
- ALLSOP, K. & STICKLER, D. J. 1985. An assessment of *Bacteroides fragilis* group organisms as indicators of human faecal pollution. *Journal of Applied microbiology*, 58, 95-99.
- ALONSO, J. L., AMOROS, I., CHONG, S. & GARELICK, H. 1996. Quantitative determination of *Escherichia coli* in water using CHROMagar® *E. coli*. *Journal of Microbiological Methods*, 25, 309-315.
- ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W. & LIPMAN, D. J. 1990. Basic local alignment search tool. *Journal of Molecular Biology*, 215, 403-410.
- ANDERSON, K. L., WHITLOCK, J. E. & HARWOOD, V. J. 2005. Persistence and Differential Survival of Fecal Indicator Bacteria in Subtropical Waters and Sediments. *Applied and environmental microbiology*, 71, 3041-3048.
- ANDERSON, W. B., HUCK, P. M., DIXON, D. G. & MAYFIELD, C. I. 2003. Endotoxin Inactivation in Water by Using Medium-Pressure UV Lamps. *Applied and environmental microbiology*, 69, 3002-3004.
- APHA 1995. Standard methods for the examination of water and wastewater. *In*: AMERICAN PUBLIC HEALTH ASSOCIATION, W., D.C (ed.) 19th Edition ed.
- ASHBOLT, N. J. 2004. Microbial contamination of drinking water and disease outcomes in developing regions. *Toxicology*, 198, 229-238.
- ATIS, S., TUTLUOGLU, B., LEVENT, E., OZTURK, C., TUNACI, A., SAHIN, K., SARAL, A., OKTAY, I., KANIK, A. & NEMERY, B. 2005. The respiratory effects of occupational polypropylene flock exposure. *European Respiratory Journal*, 25, 110-117.

- ATLAS, R. M. 1997. *Handbook of Microbiological Media*, CRC Press.
- BAGGIOLINI, M. & CLARK-LEWIS, I. 1992. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Letters*, 307, 97-101.
- BAGGIOLINI, M., WALZ, A. & KUNKEL, S. L. 1989. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *The Journal of Clinical Investigation*, 84, 1045-1049.
- BALARAJAN, R., RALEIGH, V. S., YUEN, P., WHEELER, D., MACHIN, D. & CARTWRIGHT, R. 1991. Health risks associated with bathing in sea water. *British medical journal*, 7; 303(6815), 1444-1445.
- BALLESTÉ, E. & BLANCH, A. R. 2010. Persistence of *Bacteroides* Species Populations in a River as Measured by Molecular and Culture Techniques. *Applied and environmental microbiology*, 76, 7608-7616.
- BAMFORD, S., RYLEY, H. & JACKSON, S. K. 2007. Highly purified lipopolysaccharides from Burkholderia cepacia complex clinical isolates induce inflammatory cytokine responses via TLR4-mediated MAPK signalling pathways and activation of NFκB. *Cellular Microbiology*, 9, 532-543.
- BANG, F. B. 1956. A bacterial disease of Limulus polyphemus. Bull. Johns Hopkins Hosp. 98:325.
- BARON, S. 1996. *Medical microbiology*, University of Texas Medical Branch at Galveston, 4th edition.
- BECKER, S., MUNDANDHARA, S., DEVLIN, R. B. & MADDEN, M. 2005. Regulation of cytokine production in human alveolar macrophages and airway epithelial cells in response to ambient air pollution particles: Further mechanistic studies. *Toxicology and Applied Pharmacology*, 207, 269-275.
- BERGER, F. 2004. The interleukin-6 gene: a susceptibility factor that may contribute to racial and ethnic disparities in breast cancer mortality. *Breast Cancer Research and Treatment*, 88, 281-285.
- BERGERON, P., OUJATI, H., CATALÁN CUENCA, V., HUGUET MESTRE, J. M. & COURTOIS, S. 2011. Rapid monitoring of *Escherichia coli* and *Enterococcus* spp. in bathing water using Reverse Transcription-quantitative PCR. *International Journal of Hygiene and Environmental Health*, 214, 478-484.
- BERGEY, D. H. & HOLT, J. G. 1994. *Bergey's manual of determinative bacteriology*, Baltimore, Williams & Wilkins.

- BERNHARD, A. E. & FIELD, K. G. 2000a. Identification of Nonpoint Sources of Fecal Pollution in Coastal Waters by Using Host-Specific 16S Ribosomal DNA Genetic Markers from Fecal Anaerobes. *Applied and environmental microbiology*, 66, 1587-1594.
- BERNHARD, A. E. & FIELD, K. G. 2000b. A PCR Assay To Discriminate Human and Ruminant Feces on the Basis of Host Differences in *Bacteroides-Prevotella* Genes Encoding 16S rRNA. *Applied and environmental microbiology*, 66, 4571-4574.
- BODET, C. & GRENIER, D. 2010. Synergistic effects of lipopolysaccharides from periodontopathic bacteria on pro-inflammatory cytokine production in an ex vivo whole blood model. *Molecular oral microbiology*, 25, 102-111.
- BORREGO, J. J., ARRABA, L. F., DE VICENTE, V., GÓMEZ, L. F. & ROMERO, P. 1983. Study of microbial inactivation in the marine environment. *Journal of the Water Pollution Control Federation* 297–302.
- BRADLAW, J. A. 1986. Evaluation of drug and chemical toxicity with cell culture systems. *Fundamental and Applied Toxicology*, 6, 598-606.
- BRADLEY, G. & HANCOCK, C. 2003. Increased risk of non-seasonal and body immersion recreational marine bathers contacting indicator microorganisms of sewage pollution. *Marine Pollution Bulletin*, 46, 791-794.
- BUSHON, R. N., BRADY, A. M., LIKIRDOPULOS, C. A. & CIREDU, J. V. 2009. Rapid detection of *Escherichia coli* and *enterococci* in recreational water using an immunomagnetic separation/adenosine triphosphate technique. *Journal of Applied microbiology*, 106, 432-441.
- BWR 2008. The Bathing Water Regulations. No. 1097 Printed and published in the UK by The Stationery Office Limited, under the authority and superintendence of Carol Tullo, Controller of Her Majesty's Stationery Office and Queen's Printer of Acts of Parliament.
- CABELLI, V. J. 1983. Health Effects Criteria for Marine Recreational Waters. EPA-600/1-80-031. U.S. Environmental Protection Agency, Health Effects Laboratory, Research. New York: Triangle Park,.
- CABELLI, V. J. 1989. Swimming-associated illness and recreational water quality. *Water Science and Technology*, 21, 13-21.
- CABELLI, V. J., DUFOUR, A. P., LEVIN, M. A., MCCABE, L. J. & HABERMAN, P. W. 1979. Relationship of microbial indicators to health effects at marine bathing beaches. *American Journal of Public Health*, 69, 690-696.

- CABELLI, V. J., DUFOUR, A. P., MCCABE, L. J. & LEVIN, M. A. 1982. Swimming-associated gastroenteritis and water quality. *American Journal of Epidemiology*, 115, 606.
- CABELLI, V. J., DUFOUR, A. P., MCCABE, L. J. & LEVIN, M. A. 1983. A marine recreational water quality criterion consistent with indicator concepts and risk analysis. *Water Pollution Control Federation* 55, 1306–1314
- CARDOSO, L., ARAUJO, M., GOES, A., PACIFICO, L., OLIVEIRA, R. & OLIVEIRA, S. 2007. Polymyxin B as inhibitor of LPS contamination of *Schistosoma mansoni* recombinant proteins in human cytokine analysis. *Microbial Cell Factories*, 6, 1.
- CARSON, C. A., CHRISTIANSEN, J. M., YAMPARA-IQUISE, H., BENSON, V. W., BAFFAUT, C., DAVIS, J. V., BROZ, R. R., KURTZ, W. B., ROGERS, W. M. & FALES, W. H. 2005. Specificity of a *Bacteroides thetaiotaomicron* Marker for Human Feces. *Applied and Environmental Microbiology*, 71, 4945-4949.
- CHERN, E., BRENNER, K., WYMER, L. & HAUGLAND, R. 2009. Comparison of Fecal Indicator Bacteria Densities in Marine Recreational Waters by QPCR. *Water Quality, Exposure and Health*, 1, 203-214.
- CHEUNG, W., CHANG, K. & HUNG, R. 1991. Variations in microbial indicator densities in beach waters and healthrelated assessment of bathing water quality. *epidemiology and infection*, 106, 329–344.
- CHEUNG, W. H. S., CHANG, K. C., HUNG, R. P. & KLEEVEENS, J. W. 1990. Health effects of beach water pollution in Hong Kong. *Epidemiology and Infection*, 105(1): 139–162.
- COENYE, T. & VANDAMME, P. 2003. Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiology Letters*, 228, 45-49.
- CONVERSE, R. R., BLACKWOOD, A. D., KIRS, M., GRIFFITH, J. F. & NOBLE, R. T. 2009. Rapid QPCR-based assay for fecal *Bacteroides* spp. as a tool for assessing fecal contamination in recreational waters. *Water research*, 43, 4828-4837.
- CORBETT, S. J., RUBIN, G. L., CURRY, G. K. & KLEINBAUM, D. G. 1993. The health effects of swimming at Sydney beaches. The Sydney Beach Users Study Advisory Group. *American Journal of Public Health*, 83(12): 1701–1706.
- COYE, M. J. & GOLDOFT, M. 1989. Microbiological contamination of the ocean, and human health. *New Jersey medicine*, Jul;86(7):533-8.

- CROSSETTE, B. 1996. Hope, and pragmatism, for U.N. cities conferences., New York Times 3 June 1996:A3.
- CROWTHER, J. R. 2000. *The ELISA Guidebook*, Humana Press.
- CURTIS, J. R., PATKAR, N., XIE, A., MARTIN, C., ALLISON, J. J., SAAG, M., SHATIN, D. & SAAG, K. G. 2007. Risk of serious bacterial infections among rheumatoid arthritis patients exposed to tumor necrosis factor α antagonists. *Arthritis & Rheumatism*, 56, 1125-1133.
- DAVIDSON, K. G., BERSTEN, A. D., BARR, H. A., DOWLING, K. D., NICHOLAS, T. E. & DOYLE, I. R. 2002. Endotoxin Induces Respiratory Failure and Increases Surfactant Turnover and Respiration Independent of Alveolocapillary Injury in Rats. *American Journal of Respiratory and Critical Care Medicine*, 165, 1516-1525.
- DEACON, L., PANKHURST, L., LIU, J., DREW, G., HAYES, E., JACKSON, S., LONGHURST, J., LONGHURST, P., POLLARD, S. & TYRREL, S. 2009. Endotoxin emissions from commercial composting activities. *Environmental Health*, 8, S9.
- DECOS 1998. *Endotoxins: health based recommended occupational exposure limit.*, Rijswijk: Health Council of the Netherlands pp. 1–79.
- DIABATÉ, S., MÜLHOPT, S., PAUR, H.-R. & KRUG, H. F. 2002. Pro-inflammatory Effects in Lung Cells after Exposure to Fly Ash Aerosol via the Atmosphere or the Liquid Phase. *The Annals of Occupational Hygiene*, Vol. 46, Supplement 1, pp. 382–385, .
- DICK, L. K. & FIELD, K. G. 2004. Rapid Estimation of Numbers of Fecal *Bacteroidetes* by Use of a Quantitative PCR Assay for 16S rRNA Genes. *Applied and environmental microbiology*, 70, 5695-5697.
- DIETRICH, D., FISCHER, A., MICHEL, C. & HOEGER, S. J. 2008. Toxin mixture in cyanobacterial blooms – a critical comparison of reality with current procedures employed in human health risk assessment. In: HUDNELL, H. K. (ed.) *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs*. Springer New York.
- DINARELLO, C. A. & WOLFF, S. M. 1993. The Role of Interleukin-1 in Disease. *New England Journal of Medicine*, 328, 106-113.
- DROST, E. M., KASSABIAN, G., MEISELMAN, H. J., GELMONT, D. & FISHER, T. C. 1999. Increased Rigidity and Priming of Polymorphonuclear Leukocytes in Sepsis. *American Journal of Respiratory and Critical Care Medicine*, 159, 1696-1702.

- DUBE, P. H., REVELL, P. A., CHAPLIN, D. D., LORENZ, R. G. & MILLER, V. L. 2001. A role for IL-1 α in inducing pathologic inflammation during bacterial infection. *Proceedings of the National Academy of Sciences*, 98, 10880-10885.
- ENGVALL, E. & PERLMANN, P. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry*, 8, 871-874.
- ERRIDGE, C., PRIDMORE, A., ELEY, A., STEWART, J. & POXTON, I. R. 2004. Lipopolysaccharides of *Bacteroides fragilis*, *Chlamydia trachomatis* and *Pseudomonas aeruginosa* signal via Toll-like receptor 2. *Journal of Medical Microbiology*, 53, 735-740.
- EU. 2006. Official Journal of the European Union, 2006., 64:37-52.
- EUZÉBY, J. P. 2013. *List of Prokaryotic Names with Standing in Nomenclature (LPSN)* [Online]. Available: <http://www.bacterio.net/> [Accessed 01/06/2013].
- EVANS, T. M., SCHILLINGER, J. E. & STUART, D. G. 1978. Rapid Determination of Bacteriological Water Quality by Using Limulus Lysate. *Applied and environmental microbiology*, 35, 376-382.
- FADDIN, J. F. M. 2000. *Biochemical tests for identification of medical bacteria*, Lippincott Williams & Wilkins.
- FEJER, G., WEGNER, M. D., GYÖRY, I., COHEN, I., ENGELHARD, P., VORONOV, E., MANKE, T., RUZSICS, Z., DÖLKEN, L., PRAZERES DA COSTA, O., BRANZK, N., HUBER, M., PRASSE, A., SCHNEIDER, R., APTE, R. N., GALANOS, C. & FREUDENBERG, M. A. 2013. Nontransformed, GM-CSF-dependent macrophage lines are a unique model to study tissue macrophage functions. *Proceedings of the National Academy of Sciences*.
- FIELD, K. G. & SAMADPOUR, M. 2007. Fecal source tracking, the indicator paradigm, and managing water quality. *Water research*, 41, 3517-3538.
- FIKSDAL, L., MAKI, J. S., LACROIX, S. J. & STALEY, J. T. 1985. Survival and detection of *Bacteroides* spp., prospective indicator bacteria. *Applied and environmental microbiology*, 49, 148-150.
- FISCHETTI, V. A. & MICROBIOLOGY, A. S. F. 2000. *Gram-Positive Pathogens* ASM Press.
- FLEISHER, J., JONES, F., KAY, D., STANWELL-SMITH, R., WYER, M. & MORANO, R. 1993. Water and non-water related risk factors for

gastroenteritis among bathers exposed to sewage-contaminated marine waters. *International journal of epidemiology*, 22, 698–708.

FLEISHER, J. M., FLEMING, L. E., SOLO-GABRIELE, H. M., KISH, J. K., SINIGALLIANO, C. D., PLANO, L., ELMIR, S. M., WANG, J. D., WITHUM, K., SHIBATA, T., GIDLEY, M. L., ABDELZAHER, A., HE, G., ORTEGA, C., ZHU, X., WRIGHT, M., HOLLENBECK, J. & BACKER, L. C. 2010. The BEACHES Study: health effects and exposures from non-point source microbial contaminants in subtropical recreational marine waters. *International Journal of Epidemiology*, 39, 1291-1298.

FLEISHER, J. M., KAY, D., SALMON, R. L., JONES, F., WYER, M. D. & GODFREE, A. F. 1996. Marine waters contaminated with domestic sewage: nonenteric illnesses associated with bather exposure in the United Kingdom. *American Journal of Public Health*, 86, 1228.

FLEISHER, J. M., KAY, D., WYER, M. D. & GODFREE, A. F. 1998. Estimates of the severity of illnesses associated with bathing in marine recreational waters contaminated with domestic sewage. *International Journal of Epidemiology*, 27, 722-726.

GAUTHIER, F., NEUFELD, J. D., DRISCOLL, B. T. & ARCHIBALD, F. S. 2000. Coliform Bacteria and Nitrogen Fixation in Pulp and Paper Mill Effluent Treatment Systems. *Applied and environmental microbiology*, 66, 5155-5160.

GELDREICH, E., E. 1970. Applying bacteriological parameters to recreational water quality. *American Water Works Association*, Vol. 62, No. 2, pp. 113-120.

GERBA, C. P. 2000. Indicator Microorganisms. In: MAIER, R. M. & PEPPER, I. L. (eds.) *Environmental Microbiology*. San Diego, California.: Academic Press.

GILLESPIE, S. & HAWKEY, P. M. 2006. *Principles and Practice of Clinical Bacteriology*, Wiley.

GINGER, L. C., JONATHAN, W., FELICIA, A. R., FAYE, G., SHAHED, I., TIINA, R., MICHAEL, L. M., PETER, S. T., DORR, G. D. & REBECCA, L. M. 2006. Mold and Endotoxin Levels in the Aftermath of Hurricane Katrina: A Pilot Project of Homes in New Orleans Undergoing Renovation. *Environmental Health Perspectives*, 114.

GLEESON, C. & GRAY, N. 1997. *The Coliform Index and waterborne disease problems of microbial drinking water assessment*, Taylor & Francis.

GLEICK, P. H. 2002. Dirty Water: Estimated Deaths from Water-Related Diseases 2000-2020. Pacific Institute Research Report.

- GODFREE, A. F., KAY, D. & WYER, M. D. 1997. Faecal streptococci as indicators of faecal contamination in water. *Journal of Applied microbiology*, 83, 110S-119S.
- GORBACH, S. L. & BARTLETT, J. G. 1974. Anaerobic Infections. *New England Journal of Medicine*, 290, 1237-1245.
- HAAS, C. N., MEYER, M. A., PALLER, M. S. & ZAPKIN, M. A. 1983. The utility of endotoxins as a surrogate indicator in potable water microbiology. *Water research*, 17, 803-807.
- HALL, B. G. 2011. *Phylogenetic Trees Made Easy: A How-to Manual*, Sinauer Associates, Incorporated.
- HARDIE, J. M. & WHILEY, R. A. 1995. The genus *Streptococcus*. In: WOOD, B. J. B. & HOLZAPFEL, W. H. (eds.) *The Genera of Lactic Acid Bacteria*. Springer US.
- HARRISON, D. 2012. *A Student's Guide to the Seashore*, Emerald Group Publishing Limited.
- HELYES, Z. & HAJNA, Z. 2012. Endotoxin-Induced Airway Inflammation and Asthma Models. In: SZALLASI, A. & BÍRÓ, T. (eds.) *TRP Channels in Drug Discovery*. Humana Press.
- HILGENKAMP, K. 2005. *Environmental Health: Ecological Perspectives*, Jones and Bartlett Publishers.
- HMSO. 1982. *Membrane Filtration methods* [Online]. Available: <http://www.ungiwg.org/openwater/?q=node/375>.
- HOFSTAD, T. O. R. 1975. O-antigenic specificity of lipopolysaccharides from *Bacteroides fragilis*. *Acta Pathologica Microbiologica Scandinavica Section B Microbiology*, 83B, 477-481.
- HOLST, O., ULMER, A. J., BRADE, H., FLAD, H.-D. & RIETSCHEL, E. T. 1996. Biochemistry and cell biology of bacterial endotoxins. *FEMS Immunology & Medical Microbiology*, 16, 83-104.
- HULTIN, K. A. H., KREJCI, R., PINHASSI, J., GOMEZ-CONSARNAU, L., MÅRTENSSON, E. M., HAGSTRÖM, Å. & NILSSON, E. D. 2011. Aerosol and bacterial emissions from Baltic Seawater. *Atmospheric Research*, 99, 1-14.
- IM, E., RIEGLER, F. M., POTHOUKAKIS, C. & RHEE, S. H. 2012. Elevated lipopolysaccharide in the colon evokes intestinal inflammation,

aggravated in immune modulator-impaired mice. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 303, G490-G497.

ISOGAI, H., ISOGAI, E., WAKIZAKA, H., MIURA, H., TAKANO, K., HAYASHI, M. & AND NAMIOKA, S. 1989. Effect of Lipopolysaccharide from *Bacteroides gingivalis* in SUS Rats. *Japanese journal of veterinary science*, Oct;51, (5):1095-7.

IWASAKI, A. & MEDZHITOV, R. 2010. Regulation of Adaptive Immunity by the Innate Immune System. *Science*, 327, 291-295.

JACKSON, S. K., ABATE, W., PARTON, J., JONES, S. & HARWOOD, J. L. 2008. Lysophospholipid metabolism facilitates Toll-like receptor 4 membrane translocation to regulate the inflammatory response. *Journal of Leukocyte Biology*, 84, 86-92.

JANSSENS, S. & BEYAERT, R. 2003. Role of Toll-Like Receptors in Pathogen Recognition. *Clinical Microbiology Reviews*, 16, 637-646.

JORGENSEN, J. H., CARVAJAL, H. F., CHIPPS, B. E. & SMITH, R. F. 1973. Rapid Detection of Gram-Negative Bacteriuria by Use of the Limulus Endotoxin Assay. *Applied Microbiology*, 26, 38-42.

JORGENSEN, J. H., LEE, J. C., ALEXANDER, G. A. & WOLF, H. W. 1979. Comparison of Limulus assay, standard plate count, and total coliform count for microbiological assessment of renovated wastewater. *Applied and environmental microbiology*, 37, 928-931.

JORGENSEN, J. H., LEE, J. C. & PAHREN, H. R. 1976. Rapid detection of bacterial endotoxins in drinking water and renovated wastewater. *Applied and environmental microbiology*, 32, 347-351.

JORIS, F., MANSHIAN, B. B., PEYNSHAERT, K., DE SMEDT, S. C., BRAECKMANS, K. & SOENEN, S. J. 2013. Assessing nanoparticle toxicity in cell-based assays: influence of cell culture parameters and optimized models for bridging the in vitro-in vivo gap. *Chemical Society Reviews*, 42, 8339-8359.

JOSEPH, G. S. & JULIAN, P. S. 1970. Septicemia with *Bacteroides* in patients with malignant disease. *Cancer*, 25, 663-671.

JOUSIME-SOMERS, H., SUMMANEN, P., CITRON, D. M., BELMONT, C. & WADSWORTH-KTL 2002. *Anaerobic Bacteriology Manual*, Belmont, CA, Star Publishing.

KAO, S.-J., SU, C.-F., LIU, D. D. & CHEN, H. I. 2007. Endotoxin-induced acute lung injury and organ dysfunction are attenuated by pentobarbital

anaesthesia. *Clinical and Experimental Pharmacology and Physiology*, 34, 480-487.

KASPER, D. L. 1976. Chemical and Biological Characterization of the Lipopolysaccharide of *Bacteroides fragilis* Subspecies *fragilis*. *Journal of Infectious Diseases*, 134, 59-66.

KAY, D., FLEISHER, J. M., SALMON, R., JONES, F., WYER, M. D., GODFREE, A. F., ZELENAUCH-JACQUOTTE, Z. & SHORE, R. 1994. Predicting likelihood of gastroenteritis from sea bathing: results from randomised exposure. *The Lancet*, 344, 905-909.

KEATING, S. E., MALONEY, G. M., MORAN, E. M. & BOWIE, A. G. 2007. IRAK-2 Participates in Multiple Toll-like Receptor Signaling Pathways to NF κ B via Activation of TRAF6 Ubiquitination. *Journal of Biological Chemistry*, 282, 33435-33443.

KELETI, G., SYKORA, J. L., LIPPY, E. C. & SHAPIRO, M. A. 1979. Composition and biological properties of lipopolysaccharides isolated from *Schizothrix calcicola* (Ag.) Gomont (Cyanobacteria). *Applied and environmental microbiology*, 38, 471-477.

KIEFER, D. & ALI-AKBARIAN, L. 2004. A brief evidence-based review of two gastrointestinal illnesses: irritable bowel and leaky gut syndromes. *Alternative Therapies In Health And Medicine*, 10, (3):22-30.

KILDARE, B. J., LEUTENEGGER, C. M., MCSWAIN, B. S., BAMBIC, D. G., RAJAL, V. B. & WUERTZ, S. 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: A Bayesian approach. *Water research*, 41, 3701-3715.

KIM, H. M., PARK, B. S., KIM, J.-I., KIM, S. E., LEE, J., OH, S. C., ENKHBAYAR, P., MATSUSHIMA, N., LEE, H., YOO, O. J. & LEE, J.-O. 2007. Crystal Structure of the TLR4-MD-2 Complex with Bound Endotoxin Antagonist Eritoran. *Cell*, 130, 906-917.

KINDT, T., OSBORNE, B. & GOLDSBY, R. 2006. *Kuby Immunology*, W. H. Freeman.

KODAMA, S., DAVIS, M. & FAUSTMAN, D. L. 2005. The therapeutic potential of tumor necrosis factor for autoimmune disease: a mechanistically based hypothesis. *Cellular and Molecular Life Sciences CMLS*, 62, 1850-1862.

KRASIKOVA, I. N., KAPUSTINA, N. V., ISAKOV, V. V., DMITRENOK, A. S., DMITRENOK, P. S., GORSHKOVA, N. M. & SOLOV'EVA, T. F. 2004. Detailed structure of lipid A isolated from lipopolysaccharide from the

marine proteobacterium *Marinomonas vaga* ATCC 27119T. *European Journal of Biochemistry*, 271, 2895-2904.

KREADER, C. A. 1995. Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. *Applied and environmental microbiology*, 61, 1171-1179.

KRISTIANSEN, O. P. & MANDRUP-POULSEN, T. 2005. Interleukin-6 and Diabetes: The Good, the Bad, or the Indifferent? *Diabetes*, 54, S114-S124.

LAEMMLI, U. K. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, 227, 680-685.

LAKATOS, P., FOLDES, J., HORVATH, C., KISS, L., TATRAI, A., TAKACS, I., TARJAN, G. & STERN, P. H. 1997. Serum Interleukin-6 and Bone Metabolism in Patients with Thyroid Function Disorders. *Journal of Clinical Endocrinology & Metabolism*, 82, 78-81.

LANE, D. J. 1991. 16S/23S rRNA sequencing. In: ERKO STACKEBRANDT, M. G. (ed.) *Nucleic acid techniques in bacterial systematics*. New York: John Wiley and Sons.

LAYTON, A., MCKAY, L., WILLIAMS, D., GARRETT, V., GENTRY, R. & SAYLER, G. 2006. Development of *Bacteroides* 16S rRNA Gene TaqMan-Based Real-Time PCR Assays for Estimation of Total, Human, and Bovine Fecal Pollution in Water. *Applied and environmental microbiology*, 72, 4214-4224.

LEE, J. A., JOHNSON, J. C., REYNOLDS, S. J. & THORNE, P. S. 2006. Indoor and outdoor air quality assessment of four wastewater treatment plants. *Journal of Occupational and Environmental Hygiene*, 3, (1): 36-43.

LEEMING, R., BALL, A., ASHBOLT, N. & NICHOLS, P. 1996. Using faecal sterols from humans and animals to distinguish faecal pollution in receiving waters. *Water research*, 30, 2893-2900.

LEONE, S., SILIPO, A., NAZARENKO, E. L., LANZETTA, R., PARRILLI, M. & MOLINARO, A. 2007. Molecular Structure of Endotoxins from Gram-negative Marine Bacteria: An Update. *Marine Drugs*, 5(3): , 85–112.

LEVIN, J. & BANG, F. B. 1964. The role of endotoxin in the extracellular coagulation of *Limulus* blood. *Bull. Johns Hopkins Hosp.* 115:265

LIANG, Z., HE, Z., ZHOU, X., POWELL, C. A., YANG, Y., HE, L. M. & STOFFELLA, P. J. 2013. Impact of mixed land-use practices on the

microbial water quality in a subtropical coastal watershed. *Science of The Total Environment*, 449, 426-433.

LIANG, Z., HE, Z., ZHOU, X., POWELL, C. A., YANG, Y., ROBERTS, M. G. & STOFFELLA, P. J. 2012. High diversity and differential persistence of fecal *Bacteroidales* population spiked into freshwater microcosm. *Water research*, 46, 247-257.

LIEBERS, V., BRÜNING, T. & RAULF-HEIMSOTH, M. 2006. Occupational endotoxin-exposure and possible health effects on humans (review). *American Journal of Industrial Medicine*, 49, 474-491.

LIESIVUORI, J., KOTIMAA, M., LAITINEN, S., LOUHELAINEN, K. S., PÖNNI, J., SARANTILA, R. & HUSMAN, K. 1994. Airborne endotoxin concentrations in different work conditions. *American Journal of Industrial Medicine*, 25, 123-124.

LINDBERG, A. A., WEINTRAUB, A., ZÄHRINGER, U. & RIETSCHER, E. T. 1990. Structure-Activity Relationships in Lipopolysaccharides of *Bacteroides fragilis*. *Review of Infectious Diseases*, 12, S133-S141.

LIU, J., PANKHURST, L. J., DEACON, L. J., ABATE, W., HAYES, E. T., DREW, G. H., LONGHURST, P. J., POLLARD, S., LONGHURST, J., TYRREL, S. F. & JACKSON, S. K. 2011. Evaluation of inflammatory effects of airborne endotoxin emitted from composting sources. *Environmental Toxicology and Chemistry*, 30, 602-606.

LIVINGSTON, S. J., KOMINOS, S. D. & YEE, R. B. 1978. New medium for selection and presumptive identification of the *Bacteroides fragilis* group. *Journal of Clinical Microbiology*, 7, 448-453.

LUCENA, F., LASOBRAS, J., MCINTOSH, D., FORCADELL, M. & JOFRE, J. 1994. Effect of distance from the polluting focus on relative concentrations of *Bacteroides fragilis* phages and coliphages in mussels. *Applied and environmental microbiology*, 60, 2272-2277.

MADDEN, T. 2002. The BLAST Sequence Analysis Tool. In: MCENTYRE J, O. J. (ed.) *The NCBI Handbook*. [Internet]: Bethesda (MD): National Center for Biotechnology Information (US); 2002 Chapter 16. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK21097/>.

MADIGAN, M. T., MARTINKO, J. M. & PARKER, J. 2000. *Brock Biology of Microorganisms*, Prentice Hall.

MARKHALI, F. S. 2009 *Microbiology Analysis of Drinking Water* [Online]. Articles base, Free Online Articles Directory Available: <http://www.articlesbase.com/science-articles/microbiology-analysis-of-drinking-water-1527611.html> [Accessed 10/Jan 2010].

- MARZOCCO, S., DI PAOLA, R., RIBECCO, M. T., SORRENTINO, R., DOMENICO, B., GENESIO, M., PINTO, A., AUTORE, G. & CUZZOCREA, S. 2004. Effect of methylguanidine in a model of septic shock induced by LPS. *Free radical research*, 38, 1143-1153.
- MASKELL, J. P. 1991. The resolution of bacteroides lipopolysaccharides by polyacrylamide gel electrophoresis. *Journal of Medical Microbiology*, 34, 253-257.
- MASKELL, J. P. 1994. Electrophoretic analysis of the lipopolysaccharides of *Bacteroides* spp. *Antonie van Leeuwenhoek*, 65, 155-161.
- MICHEL, O. 2000. Systemic and local airways inflammatory response to endotoxin. *Toxicology*, 152, 25-30.
- MILLER, M. J. 2013. *Rapid Test Aims for Quicker Notice of Beach Water Bacteria Levels* [Online]. Available: http://blog.rapidmicromethods.com/2013/09/rapid-test-aims-for-quicker-notice-of.html?goback=%2Egde_144585_member_270139605#%21 [Accessed 02/09/2013 2013].
- MILTON, D. K., AMSEL, J., REED, C. E., ENRIGHT, P. L., BROWN, L. R., AUGHENBAUGH, G. L. & MOREY, P. R. 1995. Cross-sectional follow-up of a flu-like respiratory illness among fiberglass manufacturing employees: endotoxin exposure associated with two distinct sequelae. *American Journal of Industrial Medicine*, 28, 469-488.
- MORENO, L. I., MILLS, D. K., ENTRY, J., SAUTTER, R. T. & MATHEE, K. 2006. Microbial Metagenome Profiling Using Amplicon Length Heterogeneity-Polymerase Chain Reaction Proves More Effective Than Elemental Analysis in Discriminating Soil Specimens*. *Journal of Forensic Sciences*, 51, 1315-1322.
- MORRIS, J. G. & ACHESON, D. 2003. Cholera and Other Types of Vibriosis: A Story of Human Pandemics and Oysters on the Half Shell. *Clinical Infectious Diseases*, 37, 272-280.
- MUELLER-SPITZ, S. R., STEWART, L. B., KLUMP, J. V. & MCLELLAN, S. L. 2010. Freshwater Suspended Sediments and Sewage Are Reservoirs for Enterotoxin-Positive *Clostridium perfringens*. *Applied and environmental microbiology*, 76, 5556-5562.
- NAKAJIMA, Y., UEDA, H., TAKEUCHI, S. & FUJIMOTO, Y. 1987. The effects of *Escherichia coli* endotoxin as a trigger for hepatic infection of rabbits with *Fusobacterium necrophorum*. *Journal of Comparative Pathology*, 97, 207-215.

- NISHIMOTO, N. 2006. Interleukin-6 in rheumatoid arthritis. *Current Opinion in Rheumatology*, 18, 277-281 10.1097/01.bor.0000218949.19860.d1.
- NIXON, W. 1995. Coastal marine eutrophication-A definition, social causes, and future concerns. *Ophelia*, 41, 199-219.
- NORWALK, C. & APPLETON, L. 1995. Indigenous microbial flora of humans. *In: Surgical Infectious Diseases*.
- NPDES. 2006. *Construction Site Stormwater Runoff Control* [Online]. National Pollutant Discharge Elimination System Available: http://cfpub.epa.gov/npdes/stormwater/menuofbmps/index.cfm?action=in_measure&min_measure_id=4 [Accessed 2/8/2010].
- NÜSSLEIN-VOLHARD, C. & WIESCHAUS, E. 1980. Mutations affecting segment number and polarity in Drosophila. *Nature*, 30;287(5785):795-801.
- O'NEILL, L. A. J. & DINARELLO, C. A. 2000. The IL-1 receptor/toll-like receptor superfamily: crucial receptors for inflammation and host defense. *Immunology Today*, 21, 206-209.
- OHREL, R. & REGISTER, K. 2002. Volunteer Estuary Monitoring A Methods Manual Available: <http://www.epa.gov/nep/monitor/pdf/manual.pdf> [Accessed 07/01/2010].
- OKABE, S. & SHIMAZU, Y. 2007. Persistence of host-specific *Bacteroides-Prevotella* 16S rRNA genetic markers in environmental waters: effects of temperature and salinity. *Applied Microbiology and Biotechnology*, 76, 935-944.
- ORMAN, K. L. & ENGLISH, B. K. 2000. Effects of Antibiotic Class on the Macrophage Inflammatory Response to Streptococcus pneumoniae. *Journal of Infectious Diseases*, 182, 1561-1565.
- OWEN, J., PUNT, J. & STRANFORD, S. 2013. *Kuby Immunology*, W. H. Freeman.
- PALCHAK, R. B., COHEN, R., AINSLIE, M. & HOERNER, C. L. 1988. Airborne Endotoxin Associated with Industrial-Scale Production of Protein Products in Gram-Negative Bacteria. *American Industrial Hygiene Association Journal*, 49, 420-421.
- PALMER, J. D. & RIFKIND, D. 1974. Neutralization of the hemodynamic effects of endotoxin by polymyxin B. *The Journal of surgery, gynecology and obstetrics* [Online], 138.

- PANKHURST, L. J., DEACON, L. J., LIU, J., DREW, G. H., HAYES, E. T., JACKSON, S., LONGHURST, P. J., LONGHURST, J. W. S., POLLARD, S. J. T. & TYRREL, S. F. 2011. Spatial variations in airborne microorganism and endotoxin concentrations at green waste composting facilities. *International Journal of Hygiene and Environmental Health*, 214, 376-383.
- PÉTERFI, Z. & KOCSIS, B. 2000. Comparison of Blocking Agents for an Elisa for Lps. *Journal of Immunoassay*, 21, 341-354.
- PINET, P. R. 2009. *Invitation to oceanography*, Jones & Bartlett Publishers.
- PITTOIS, S., JACKSON, M. H. & WOOD, B. J. B. 2000. Problems associated with the presence of cyanobacteria in recreational and drinking waters. *International Journal of Environmental Health Research*, 10, 203-218.
- POLTORAK, A., HE, X., SMIRNOVA, I., LIU, M.-Y., HUFFEL, C. V., DU, X., BIRDWELL, D., ALEJOS, E., SILVA, M., GALANOS, C., FREUDENBERG, M., RICCIARDI-CASTAGNOLI, P., LAYTON, B. & BEUTLER, B. 1998. Defective LPS Signaling in C3H/HeJ and C57BL/10ScCr Mice: Mutations in Tlr4 Gene. *Science*, 282, 2085-2088.
- POND, K. 2005. Water Recreation and Disease *In*: DATA, W. L. C.-I.-P. (ed.). London. Seattle: IWA Publishing.
- POXTON, I. R. & EDMOND, D. M. 1995. Biological Activity of *Bacteroides* Lipopolysaccharide: Reappraisal *Clinical Infectious Diseases*, 20, S149-S153
- PRIETO, M. D., LOPEZ, B., JUANES, J. A., REVILLA, J. A., LLORCA, J. & DELGADO-RODRÍGUEZ, M. 2001. Recreation in coastal waters: health risks associated with bathing in sea water. *Journal of Epidemiology and Community Health*, 55, 442-447.
- RAETZ, C. R. H. 1990. Biochemistry of Endotoxins. *Annual Review of Biochemistry*, 59, 129-170.
- RAETZ, C. R. H., GUAN, Z., INGRAM, B. O., SIX, D. A., SONG, F., WANG, X. & ZHAO, J. 2009. Discovery of new biosynthetic pathways: the lipid A story. *Journal of Lipid Research*, 50, S103-S108.
- RAETZ, C. R. H. & WHITFIELD, C. 2002. LIPOPOLYSACCHARIDE ENDOTOXINS. *Annual Review of Biochemistry*, 71, 635-700.
- RAINA M. MAIER, IAN L. PEPPER & GERBA, C. P. 2009. Environmental microbiology *Environmental microbiology* Edition: 2 ed.

- RAMOS, J. L., GALLEGOS, M. A.-T., MARQUÉS, S., RAMOS-GONZÁLEZ, M.-I., ESPINOSA-URGEL, M. & SEGURA, A. 2001. Responses of Gram-negative bacteria to certain environmental stressors. *Current Opinion in Microbiology*, 4, 166-171.
- REISCHER, G. H., KASPER, D. C., STEINBORN, R., FARNLEITNER, A. H. & MACH, R. L. 2007. A quantitative real-time PCR assay for the highly sensitive and specific detection of human faecal influence in spring water from a large alpine catchment area. *Letters in Applied Microbiology*, 44, 351-356.
- RHODES, M. W. & AND KATOR, H. I. 1991. Use of *Salmonella typhimurium* WG49 to enumerate male -specific coliphages in as estuary and watershed subject to nonpoint pollution. *Water Research*, 25, 1315-1323.
- RICE, J. B., STOLL, L. L., LI, W.-G., DENNING, G. M., WEYDERT, J., CHARIPAR, E., RICHENBACHER, W. E., MILLER, F. J. & WEINTRAUB, N. L. 2003. Low-Level Endotoxin Induces Potent Inflammatory Activation of Human Blood Vessels: Inhibition by Statins. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 23, 1576-1582.
- RIETSCHEL, E. T., BRADE, H., HOLST, O., BRADE, L., MÜLLER-LOENNIES, S., MAMAT, U., ZÄHRINGER, U., BECKMANN, F., SEYDEL, U., BRANDENBURG, K., ULMER, A. J., MATTERN, T., HEINE, H., SCHLETTER, J., LOPPNOW, H., SCHÖNBECK, U., FLAD, H. D., HAUSCHILDT, S., SCHADE, U. F., PADOVA, F., KUSUMOTO, S. & SCHUMANN, R. R. 1996. Bacterial Endotoxin: Chemical Constitution, Biological Recognition, Host Response, and Immunological Detoxification. In: RIETSCHEL, E. T. & WAGNER, H. (eds.) *Pathology of Septic Shock*. Springer Berlin Heidelberg.
- RIETSCHEL, E. T., BRADE, L., LINDNER, B. & ZÄHRINGER, U. 1992. Biochemistry of lipopolysaccharides. In: MORRISON, D. C. & RYAN, J. L. (eds.) *Bacterial endotoxic lipopolysaccharide*. Press, Boca Raton .
- RIETSCHEL, E. T., KIRIKAE, T., SCHADE, F. U., MAMAT, U., SCHMIDT, G., LOPPNOW, H., ULMER, A. J., ZÄHRINGER, U., SEYDEL, U. & DI PADOVA, F. 1994. Bacterial endotoxin: molecular relationships of structure to activity and function. *The FASEB Journal*, 8, 217-25.
- RIETSCHEL, E. T., KIRIKAE, T., SCHADE, F. U., ULMER, A. J., HOLST, O., BRADE, H., SCHMIDT, G., MAMAT, U., GRIMMECKE, H.-D., KUSUMOTO, S. & ZÄHRINGER, U. 1993. The chemical structure of bacterial endotoxin in relation to bioactivity. *Immunobiology*, 187, 169-190.
- ROUND, J. L., LEE, S. M., LI, J., TRAN, G., JABRI, B., CHATILA, T. A. & MAZMANIAN, S. K. 2011. The Toll-Like Receptor 2 Pathway Establishes

Colonization by a Commensal of the Human Microbiota. *Science*, 332, 974-977.

RYAN, K. J. 2004. *Sherris medical microbiology: an introduction to infectious diseases* Appleton & Lange.

SAKAMOTO, M. & BENNO, Y. 2006. Reclassification of *Bacteroides distasonis*, *Bacteroides goldsteinii* and *Bacteroides merdae* as *Parabacteroides distasonis* gen. nov., comb. nov., *Parabacteroides goldsteinii* comb. nov. and *Parabacteroides merdae* comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, 56, 1599-1605.

SANDSTROM, T., BJERMER, L. & RYLANDER, R. 1992. Lipopolysaccharide (LPS) inhalation in healthy subjects increases neutrophils, lymphocytes and fibronectin levels in bronchoalveolar lavage fluid. *European Respiratory Journal*, 5, 992-996.

SANGER, F., NICKLEN, S. & COULSON, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, 74, 5463-5467.

SCHLEEF, R. R., KENNEY, D. M. & SHEPRO, D. 1979. Effect of sodium chloride on limulus amebocyte lysate. Inhibition of endotoxin activation of procoagulase. *Thrombosis and haemostasis*, 41, 329-336.

SCHULTZ, C. L., BURET, A. G., OLSON, M. E., CERI, H., READ, R. R. & MORCK, D. W. 2000. Lipopolysaccharide Entry in the Damaged Cornea and Specific Uptake by Polymorphonuclear Neutrophils. *Infection and Immunity*, 68, 1731-1734.

SCHÜTTE, H., ROSSEAU, S., CZYMEK, R., ERMERT, L., WALMRATH, D., KRÄMER, H.-J., SEEGER, W. & GRIMMINGER, F. 1997. Synergism Between Endotoxin Priming and Exotoxin Challenge in Provoking Severe Vascular Leakage in Rabbit Lungs. *American Journal of Respiratory and Critical Care Medicine*, 156, 819-824.

SCOGING, A. C. 1991. Illness associated with seafood. *CDR, London, England: Review*, 11;1, (11):R117-22.

SCOTT, T. M., ROSE, J. B., JENKINS, T. M., FARRAH, S. R. & LUKASIK, J. 2002. Microbial Source Tracking: Current Methodology and Future Directions. *Applied and environmental microbiology*, 68, 5796-5803.

SEPA 2002. Scottish Bathing Waters: ISBN 1 901 322 41 6.

- SEPP, R., SZABO, I., UDA, H. & SAKAMOTO, H. 1994. Rapid techniques for DNA extraction from routinely processed archival tissue for use in PCR. *Journal of clinical pathology*, 47, 318-23.
- SHAPIRA, L., SOSKOLNE, W. A., HOURI, Y., BARAK, V., HALABI, A. & STABHOLZ, A. 1996. Protection against endotoxic shock and lipopolysaccharide-induced local inflammation by tetracycline: correlation with inhibition of cytokine secretion. *Infection and Immunity*, 64, 825-8.
- SHIAU, M. Y., WU, C. Y., HUANG, C. N., HU, S. W., LIN, S. J. & CHANG, Y. H. 2003. TNF- α polymorphisms and type 2 diabetes mellitus in Taiwanese patients. *Tissue Antigens*, 61, 393-397.
- SIKKA, S. C., CHAMPION, H. C., BIVALACQUA, T. J., ESTRADA, L. S., WANG, R., RAJASEKARAN, M., AGGARWAL, B. B. & HELLSTROM, W. J. G. 2001. Role of genitourinary inflammation in infertility: synergistic effect of lipopolysaccharide and interferon- γ on human spermatozoa. *International Journal of Andrology*, 24, 136-141.
- SIMPSON, J. C. G., NIVEN, R. M., PICKERING, C. A. C., OLDHAM, L. A., FLETCHER, A. M. & FRANCIS, H. C. 1999. Comparative Personal Exposures to Organic Dusts and Endotoxin. *Annals of Occupational Hygiene*, 43, 107-115.
- SINCLAIR, R. G., JONES, E. L. & GERBA, C. P. 2009. Viruses in recreational water-borne disease outbreaks: a review. *Journal of Applied microbiology*, 107, 1769-1780.
- SOLUM, N. O. 1973. The coagulogen of *Limulus polyphemus* hemocytes. A comparison of the clotted and non-clotted forms of the molecule. *Thrombosis Research*, 2, 55-69.
- SOLUM, N. O. N. 1970. Some characteristics of the clottable protein of *limulus polyphemus* blood cells. *Thromb Diath Haemorrh*, 23, 170-181.
- STEVENSON, A. 2010. *Oxford Dictionary of English*, OUP Oxford.
- STEWART, I., SCHLUTER, P. & SHAW, G. 2006. Cyanobacterial lipopolysaccharides and human health - a review. *Environmental Health: A Global Access Science Source*, 5, 7.
- SUN, L., DENG, L., EA, C.-K., XIA, Z.-P. & CHEN, Z. J. 2004. The TRAF6 Ubiquitin Ligase and TAK1 Kinase Mediate IKK Activation by BCL10 and MALT1 in T Lymphocytes. *Molecular Cell*, 14, 289-301.
- TAKADA, H., KOTANI, S., TANAKA, S., OGAWA, T., TAKAHASHI, I., TSUJIMOTO, M., KOMURO, T., SHIBA, T., KUSUMOTO, S.,

- KUSUNOSE, N., HASEGAWA, A. & KISO, M. 1988. Structural requirements of lipid A species in activation of clotting enzymes from the horseshoe crab, and the human complement cascade. *European Journal of Biochemistry*, 175, 573-580.
- TAKEDA, K. & AKIRA, S. 2004. TLR signaling pathways. *Seminars in Immunology*, 16, 3-9.
- TAMBALO, D. D., BOA, T., LILJEBJELKE, K. & YOST, C. K. 2012. Evaluation of two quantitative PCR assays using *Bacteroidales* and mitochondrial DNA markers for tracking dog fecal contamination in waterbodies. *Journal of Microbiological Methods*, 91, 459-467.
- TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*, 28, 2731-2739.
- TAO, H., BREWIN, N. J. & NOEL, K. D. 1992. *Rhizobium leguminosarum* CFN42 lipopolysaccharide antigenic changes induced by environmental conditions. *Journal of Bacteriology*, 174, 2222-2229.
- TARTERA, C. & JOFRE, J. 1987. Bacteriophages active against *Bacteroides fragilis* in sewage-polluted waters. *Applied and environmental microbiology*, 53, 1632 - 1637.
- TROUGHTON, P. R., PLATT, R., BIRD, H., EL-MANZALAWI, E., BASSIOUNI, M. & WRIGHT, V. 1996. Synovial fluid interleukin-8 and neutrophil function in rheumatoid arthritis and seronegative polyarthritis. *Rheumatology*, 35, 1244-1251.
- VENDRAMINI, A. A., DE LÁBIO, R. W., RASMUSSEN, L. T., MINETT, T., BERTOLUCCI, P. H. F., DE ARRUDA CARDOSO SMITH, M. & PAYÃO, S. L. M. 2007. Interleukin-8 Gene Polymorphism -251T>A and Alzheimer's Disease. *Journal of Alzheimer's Disease*, 12, 221-222.
- VON SCHIRNDING, Y. E., KFIR, R., CABELLI, V. J., FRANKLIN, L. & JOUBERT, G. 1992. Morbidity among bathers exposed to polluted sea water. A prospective epidemiological study. . *South African Medical Journal*, 81, 543-546.
- WAINES, P. L., MOATE, R., MOODY, A. J., ALLEN, M. & BRADLEY, G. 2011. The effect of material choice on biofilm formation in a model warm water distribution system. *Biofouling*, 27, 1161-1174.
- WATSON, S. W., NOVITSKY, T. J., QUINBY, H. L. & VALOIS, F. W. 1977. Determination of bacterial number and biomass in the marine environment. *Applied and environmental microbiology*, 33, 940-6.


- WEINTRAUB, A., ZÄHRINGER, U., WOLLENWEBER, H.-W., SEYDEL, U. & RIETSCHER, E. T. 1989. Structural characterization of the lipid A component of *Bacteroides fragilis* strain NCTC 9343 lipopolysaccharide. *European Journal of Biochemistry*, 183, 425-431.
- WOLLIN, L., UHLIG, S., NÜSING, R. & WENDEL, A. 2001. Granulocyte-Macrophage Colony-Stimulating Factor Amplifies Lipopolysaccharide-induced Bronchoconstriction by a Neutrophil- and Cyclooxygenase 2-Dependent Mechanism. *American Journal of Respiratory and Critical Care Medicine*, 163, 443-450.
- WORTHEN, G. S., HASLETT, C., REES, A. J., GUMBAY, R. S., HENSON, J. E. & HENSON, P. M. 1987. Neutrophil-mediated Pulmonary Vascular Injury: Synergistic Effect of Trace Amounts of Lipopolysaccharide and Neutrophil Stimuli on Vascular Permeability and Neutrophil Sequestration in the Lung. *American Review of Respiratory Disease*, 136, 19-28.
- YAMADA, H., MIYAZAKI, H., KIKUCHI, T., FUJIMOTO, J. & KUDOH, I. 2000. Acid Instillation Enhances the Inflammatory Response to Subsequent Lipopolysaccharide Challenge in Rats. *American Journal of Respiratory and Critical Care Medicine*, 162, 1366-1371.
- YAMAMOTO, Y., HARASHIMA, A., SAITO, H., TSUNEYAMA, K., MUNESUE, S., MOTOYOSHI, S., HAN, D., WATANABE, T., ASANO, M., TAKASAWA, S., OKAMOTO, H., SHIMURA, S., KARASAWA, T., YONEKURA, H. & YAMAMOTO, H. 2011. Septic Shock Is Associated with Receptor for Advanced Glycation End Products Ligation of LPS. *The Journal of Immunology*, 186, 3248-3257.
- YANAGISAWA, R., TAKANO, H., INOUE, K., ICHINOSE, T., SADAKANE, K., YOSHINO, S., YAMAKI, K., KUMAGAI, Y., UCHIYAMA, K., YOSHIKAWA, T. & MORITA, M. 2003. Enhancement of acute lung injury related to bacterial endotoxin by components of diesel exhaust particles. *Thorax*, 58, 605-612.
- YOSHIMURA, T., MATSUSHIMA, K., OPPENHEIM, J. J. & LEONARD, E. J. 1987. Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)-stimulated human blood mononuclear leukocytes: partial characterization and separation from interleukin 1 (IL 1). *The Journal of Immunology*, 139, 788-793.
- YOUNG, N. S., LEVIN, J. & PRENDERGAST, R. A. 1972. An invertebrate coagulation system activated by endotoxin: evidence for enzymatic mediation. *The Journal of Clinical Investigation*, 51, 1790-1797.
- ZAVASCKI, A. P., GOLDANI, L. Z., LI, J. & NATION, R. L. 2007. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *Journal of Antimicrobial Chemotherapy*, 60, 1206-1215.

- ZIEGLER-HEITBROC, H. W. L., THIEL, E., FUTTERER, A., HERZOG, V., WIRTZ, A. & RIETHMÜLLER, G. 1988. Establishment of a human cell line (mono mac 6) with characteristics of mature monocytes. *International Journal of Cancer*, 41, 456-461.
- ZUGHAIER, S. M., RYLEY, H. C. & JACKSON, S. K. 1999a. Lipopolysaccharide (LPS) from *Burkholderia cepacia* Is More Active than LPS from *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* in Stimulating Tumor Necrosis Factor Alpha from Human Monocytes. *Infection and Immunity*, 67, 1505-1507.
- ZUGHAIER, S. M., RYLEY, H. C. & JACKSON, S. K. 1999b. A Melanin Pigment Purified from an Epidemic Strain of *Burkholderia cepacia* Attenuates Monocyte Respiratory Burst Activity by Scavenging Superoxide Anion. *Infection and Immunity*, 67, 908-913.

Appendices

Appendix A

Modules Transcript

University of Plymouth Transcript									
Date: 07 July 2010					 Faculty of Science and Technology University of Plymouth Drake Circus Plymouth Devon PL4 8AA United Kingdom tel +44 (0)1752 584 584 fax +44 (0)1752 584 540 www.plymouth.ac.uk Mrs Christine Mushens BA Faculty Business Manager				
Date of Birth 05/03/1984									
Student Reference Number: 10250431									
HESA Reference: 0000102504318									
Name	Anas Akram Sattar Sattar								
Address	Building No.12/First Floor/Flat 1 Utba Street Baba Amr Homs Syria Email Anasrami84@Yahoo.Com								
Institution responsible for programme delivery: University of Plymouth									
Language(s) of Instruction/Assessment: English									
ResM Biological Sciences Stage 1 - 2009/2010									
Credit achieved in this academic year:					60				
Programme/Stage Aggregate Final Mark:									
Award Board:					24th July 2010				
Progression:					A2/ Module results and any credit awarded are as indicated on the transcript.				
Module Code	Module Title	Credit	Level	Mark (%)	Att.	Result	Mod. Decn.		
BIO5102	Principles and Applications in Electron Microscopy Coursework	10	7	65.00	1	A			
BIO5124	Postgraduate Research Skills and Methods Coursework	20	7	61.50	1	A			
BIOL3309	Marine Microbiology - Ecology and Applications Coursework Examination	20	6	54.77	1	A			
ENV5101	Laboratory Based Teaching Methods and Practice Coursework	10	7	75.00	1	A			

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Appendix B

General Teaching Associate (GTA) course



**Directorate of Teaching
and Learning**

3 Endsleigh Place
University of Plymouth
Drake Circus
Plymouth
PL4 8AA
United Kingdom

Tel: +44 (0)1752 82035/34

15th March 2010

Dear Anas,

Many thanks for joining us on the GTA 2010-1 standard course. Jennie, Joan, Terri, Polly and I very much enjoyed working with you, and appreciated all the input and discussion from the whole group.

Please find enclosed your Certificate of Professional Development. I hope you enjoyed the course and find some elements of it useful to you in practice.

Remember if you do go on to complete the accredited part of the course, LTHE 300, you will also be eligible for Associate Membership in the Higher Education Academy. Find out more at www.gtacourse.net.

With the very best of wishes,



Vivian Neal
GTA Course Lead

Anas: Thanks for your
contributions!

General Teaching Associate certificate

University of Plymouth



Certificate of Professional Development

Learning and Teaching for General Teaching Associates

A course run by Educational Development:
see www.gtacourse.net for further details

This is to certify that

Anas Sattar

**has attended the General Teaching Associates course, which included
taught sessions and online activities as detailed below**

Sessions Attended

Theories of Learning and Teaching
Presentation Skills
Evaluating Teaching
Learning in Groups
Feedback
Assessment

Online Activities

Dealing with Difficult Situations
Issues in Assessment
Reflective Learning Blogs

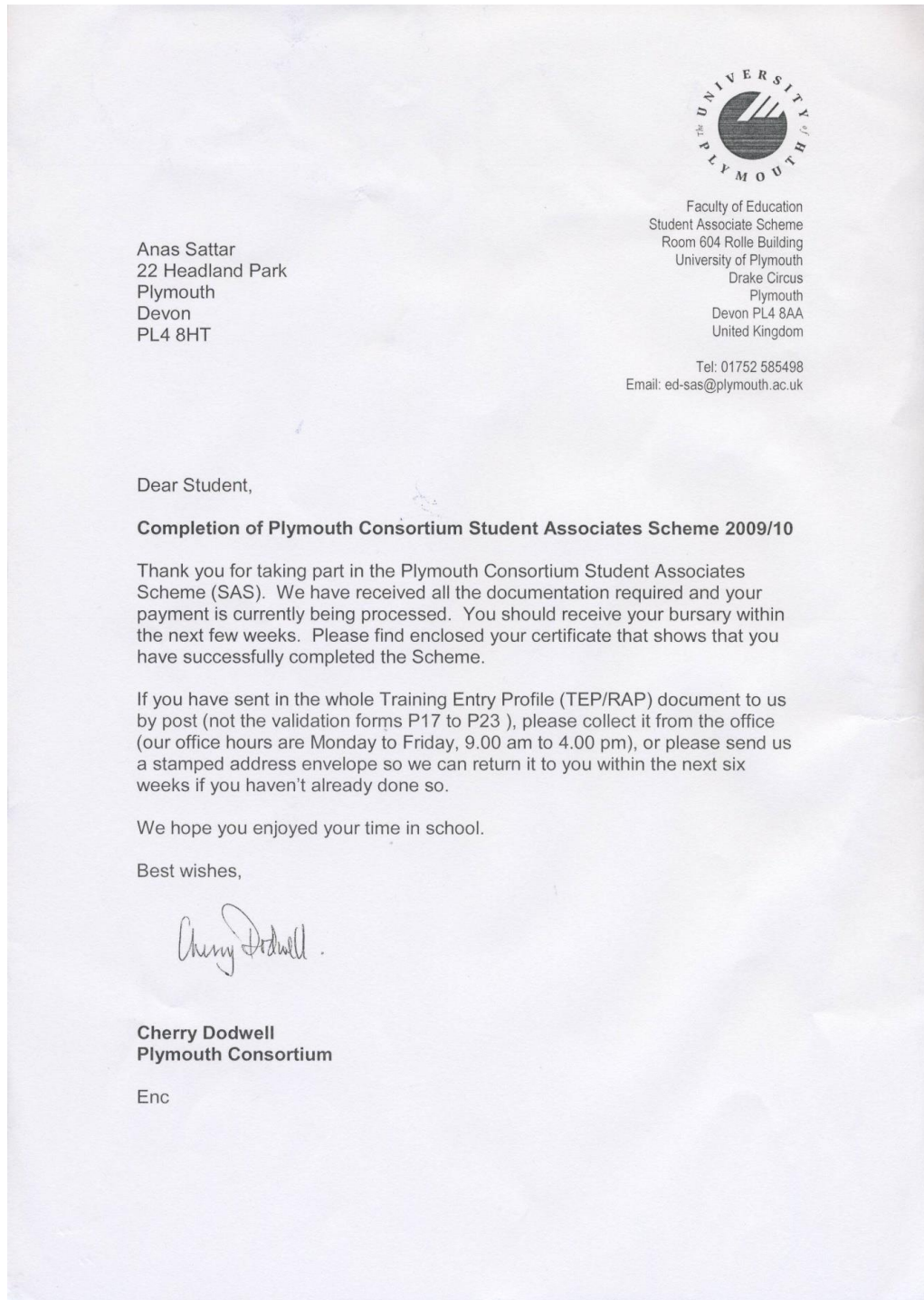
Signed: 

Vivian Neal – Academic Developer and GTA Lead

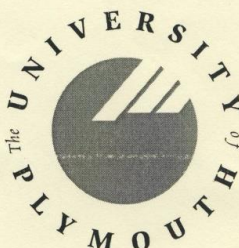
Date of issue: 15th March 2010

Appendix C

Student associate scheme (SAS) completion letter



Student associate scheme certificate



Plymouth Consortium *Student Associate Scheme*

This is to certify that

Anas Sattar

Successfully completed the Student Associate Scheme for 2009/10.

- The Scheme involved Student Associates working with pupils, teachers, parents/carers, support and other school staff.
- Student Associates worked collaboratively with individual pupils and groups providing tutorial, mentoring and coaching support under supervision.
- The Scheme introduced Student Associates to the National Curriculum, Aimhigher-Widening Participation, Special Educational Needs, Equal Opportunities, Child Development, Behaviour Management and working with Gifted and Talented pupils.
- Student Associates completed a school based work placement for fifteen days.

Project Manager
Student Associates Scheme

Appendix D

Certificate of scientific sessions attendance, ASM conference 2012, San Francisco, USA



AMERICAN
SOCIETY FOR
MICROBIOLOGY

California #0035
Florida #273

CertificateNumber:
696349C_U6918_A3942_O1_T6



112th General Meeting | American Society for Microbiology
June 16-19, 2012 | San Francisco, California

CERTIFICATE OF ATTENDANCE

Anas A Sattar

asm2012: 112th General Meeting

Saturday, June 16, 2012 - Tuesday, June 19, 2012, San Francisco,
CA

Date	Session Title	Time	Max. Credits Designated	Credits Awarded
6/17/2012	Best Practices Antimicrobial Susceptibility Testing: An Update from CLSI Program #:273-003-12	08:15 AM - 10:45 AM	2.5	2.5
6/17/2012	Diagnostic Challenges in Gastroenteritis: Emerging Methodologies Meet Emerging Pathogens Program #:273-004-12	08:15 AM - 10:45 AM	2.5	2.5
6/17/2012	New Insights in Global Surveillance of Current and Emerging Infectious Diseases Program #:273-007-12	08:15 AM - 10:45 AM	2.5	2.5
6/17/2012	In the Year 2525: Survival Strategies and Clinical Microbiology's Leadership Role in Tomorrow's Healthcare Teams Program #:273-154-12	11:00 AM - 01:30 PM	2.5	2.5
6/18/2012	Best Practices in STI Testing for HIV, Syphilis, GC and Chlamydia Program #:273-012-12	08:15 AM - 10:45 AM	2.5	2.5
6/18/2012	Diagnostic and Therapeutic Implications of Novel Mechanisms of Resistance Program #:273-013-12	08:15 AM - 10:45 AM	2.5	2.5
6/18/2012	New Technology: Is the Juice Worth the Squeeze? Program #:273-123-12	08:15 AM - 10:45 AM	2.5	2.5
6/19/2012	On Top of Outbreaks: New Rapid Next Generation Sequencing Deployed for Public Health and Clinical Microbiology Program #:273-135-12	03:00 PM - 05:30 PM	2.5	2.5
			Total Credits	20

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I certify that this individual has
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A handwritten signature in cursive script, reading "Peggy McNult".

Peggy McNult
Assistant Director, Professional
Practice
6/19/2012

ASM Professional Practice Committee | tel: 202-942-9225 | www.asm.org

Appendix E

Exhibitor Exam certificate of attendance, ASM conference 2012, San Francisco, USA



AMERICAN
SOCIETY FOR
MICROBIOLOGY

California #0035
Florida #273
Certificate Number: 368781C_U6918_A3944_O1_T6



CERTIFICATE OF ATTENDANCE

Exhibitor Exam asm2012: 112th General Meeting, (PACE # 273-153-12),
Sunday, June 17, 2012 - Tuesday, June 19, 2012, San Francisco, CA

I Anas A. Sattar attest that I have attended the sessions listed above 03/11/2012
Signature Date

The American Society for Microbiology certifies that Anas A Sattar has participated in the education activity
titled Exhibitor Exam asm2012: 112th General Meeting at San Francisco, CA on Sunday, June 17, 2012 -
Tuesday, June 19, 2012 and is awarded 2 hours of P.A.C.E.® continuing education.

This activity was designated for 2 hours of P.A.C.E.® continuing education.

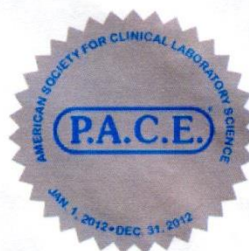
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Laboratory Scientists.*

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Peggy McNult
Assistant Director, Professional Practice
6/19/2012


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Appendix F

Poster presentation, ASM conference 2012, San Francisco, USA



Endotoxin Activity of *Bacteroides* Lipopolysaccharides from Contaminated Marine Bathing Waters

Anas Sattar, Graham Bradley, Neema Habil, Andrew Foey, Simon Jackson
School of Biomedical and Biological Sciences, Plymouth University, Drake Circus, Plymouth, Devon, PL4 8AA, UK



Bacteroides species are prominent bacteria in human and other warm blood animal faeces and are present in bathing waters when polluted with poorly processed sewage from urban sewage discharge or from run off from farms. As well as indicators of other pathogen *Bacteroides* species are also opportunistic bacteria and can cause a variety of health complications depending on their route of entry. Extracted Lipopolysaccharide (LPS) or endotoxin from *Bacteroides* species and *E. coli* were compared in their capability to stimulate Interleukin 6 (IL-6) and Tumour Necrosis Alpha (TNF- α) from the Human origin monocyte Mono Mac 6. The extracted LPS of *Bacteroides* species showed that it was approximately 100 fold less active in producing TNF- α and IL-6 than the LPS of *E. coli*. The Activity of *Bacteroides* species was also determined using Limulus Amebocyte Lysate (LAL) assay. Also, *Bacteroides* LPS was profiled using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and results showed that isolated *Bacteroides* species have a high similarity of LPS banding pattern among them lending *Bacteroides* LPS to possible use as a pollution biomarker.

Aim

This study was carried out to profile the LPS of *Bacteroides* isolated from a local marine bathing water using SDS PAGE as well as measuring endotoxin activity using Limulus Amebocyte Lysate (LAL) assay. The potency was also assessed by stimulating human monocyte Mono Mac 6 cell line and measuring two main pro-inflammatory cytokines TNF- α and IL-6.

Materials and Methods

- *Bacteroides* isolation and speciation

Bacteroides species were isolated from a Local bathing site in Plymouth UK using the Membrane Filtration Method and identified using biochemical and molecular techniques.

- LAL assay and LPS profiling

LPS activity of *Bacteroides* species was determined using Genscript Limulus Amebocyte Assay. SDS PAGE was used to profile *Bacteroides* LPS and compared to *E. coli* LPS.

- Cell culture assay

Mono Mac 6 monocytes cell lines were stimulated with 3 concentrations of *Bacteroides* LPS (1000, 100 and 10 ng/ml) and measured the production of pro-inflammatory cytokine using Sandwich Elisa. *E. coli* O111:B4 LPS was used as a control.

Conclusion

Results showed that *Bacteroides* LPS potency is significantly lower than the LPS of *E. coli* O111:B4 when used as a stimulant for MM6 cells. However *Bacteroides* species LPS showed its ability to stimulate MM6 cells to undergo an inflammatory response. The LPS activity of *Bacteroides* species is also lower than *E. coli* O111:B4 when measured using the LAL assay. LPS of *Bacteroides* species isolates were very similar when profiled using SDS PAGE, this was also confirmed with the LAL assay and showed that LPS potency is highly conserved in the species found in contaminated marine bathing water. This bodes well for the use of *Bacteroides* LPS as a biomarker of such faecal pollution.

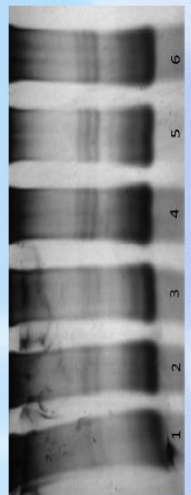


Figure 1: SDS page showing six *Bacteroides* species LPS. B. ovis 2= B. eggerthii 3= B. fragilis NCTC 6445 4= B. fragilis 5= B. uniformis 6= B. distasonis

<i>Bacteroides</i> Spp	LAL activity of 1ng of <i>Bacteroides</i> LPS in EU/ml
<i>B. fragilis</i>	0.030
<i>B. ovis</i>	0.029
<i>B. uniformis</i>	0.029
<i>B. distasonis</i>	0.030
<i>B. eggerthii</i>	0.024

Table 1: LPS activity of *Bacteroides* species using LAL assay

Results of tissue culture showed that *Bacteroides* LPS successfully stimulated Mono Mac 6 cells and led to the production of pro-inflammatory cytokines TNF- α and IL-6 measured using sandwich ELISA. Results also showed that these five *Bacteroides* LPS are about a hundred times less potent than *E. coli* O111:B4 when stimulated with 10 ng of its LPS. Bars show mean of 3 independent experiments. Statistical analysis using One Way Analysis of Variance and Tukey's test showed a significant differences among *Bacteroides* species LPS in stimulating MM6 and the production of TNF- α and IL-6.

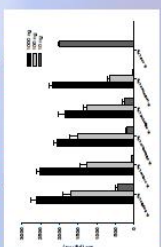


Figure 2: concentration of pro-inflammatory cytokine TNF- α (pg/ml) of purified LPS of *Bacteroides* species with a P value of <0.001

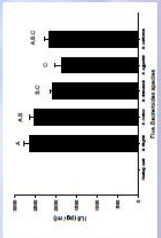


Figure 3: concentration of pro-inflammatory cytokine IL-6 (pg/ml) of purified LPS of *Bacteroides* species with a P value of <0.001

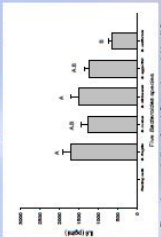


Figure 4: concentration of pro-inflammatory cytokine TNF- α (pg/ml) of purified LPS of *Bacteroides* species with a P value of <0.001

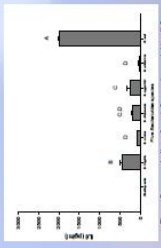


Figure 5: concentration of pro-inflammatory cytokine IL-6 (pg/ml) of purified LPS of *Bacteroides* species with a P value of <0.001

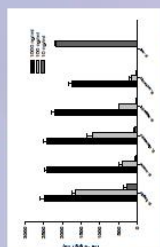


Figure 6: concentration of pro-inflammatory cytokine TNF- α (pg/ml) of purified LPS of *Bacteroides* species with a P value of <0.001

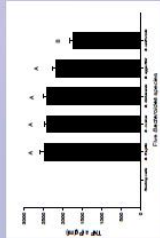


Figure 7: concentration of pro-inflammatory cytokine IL-6 (pg/ml) of purified LPS of *Bacteroides* species with a P value of <0.001

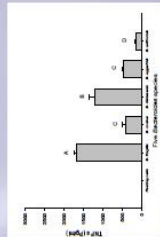


Figure 8: concentration of pro-inflammatory cytokine TNF- α (pg/ml) of purified LPS of *Bacteroides* species with a P value of <0.001

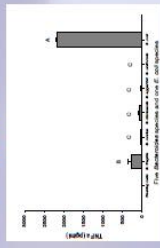


Figure 9: concentration of pro-inflammatory cytokine IL-6 (pg/ml) of purified LPS of *Bacteroides* species with a P value of <0.001

Acknowledgment

Special thanks to SFAM for funding me to attend this conference. Many thanks to Dr. Wondwossen Abate Wolde and Louise Belfield for their help.

Appendix G

Poster presentation, SFAM conference 2011, Dublin, republic of Ireland

Lipopolysaccharide as a real-time biomarker of human sewage pathogens in marine bathing waters

Anas Sattar, Graham Bradley, Simon Jackson

School Of Biomedical and Biological Sciences, University of Plymouth, United Kingdom



Introduction

Bacterial indicators of human pathogens are important for determining the quality of marine bathing waters. Current culture-based methods (*Escherichia coli* and Enterococci) are however retrospective with results taking 24-48 hours and PCR-based molecular tests (*Bacteroides*) are only useful for laboratory situations. A rapid, on-site, yes/no test needs to be developed to be used by untrained sportspeople. Lipopolysaccharide (LPS) present in Gram negative bacteria may be such a useful real-time biomarker. This study investigates the possibility of a positive/negative, rapid detection method for LPS and its use as a sewage pollution indicator.

Objectives

The objectives of this research were to use a commercial endotoxin (LPS) kit to establish the minimum detection levels for Gram negative bacteria in seawater, to establish if a pollutive rise in LPS could be detected over background levels, and to profile the LPS of local *Bacteroides* spp. seawater isolates for possible use as specific biomarkers.

Materials and Methods

Bacterial isolation and endotoxin detection.

Local seawater samples were collected and filtered using a 0.45 µm filter then placed onto selective media; *Bacteroides* Bile Esculin (BBE) and MLSB (*E. coli*). A marine Gram negative bacterium was also isolated on seawater agar. Bacterial cells were harvested by centrifugation after overnight broth culture.

A commercial endotoxin detection kit (Genscript, USA) was used to measure the minimum level of bacterial LPS from *Bacteroides*, *E. coli* and Gram negative bacterial isolates that could be detected in endotoxin-free water and seawater.

***Bacteroides* LPS profiling.**

Six *Bacteroides* spp isolates were identified using biochemical and sugar utilisation tests profiling the species commonly found in seawater samples. Confirmation using DNA isolation and 16S rRNA sequencing was attempted. These isolates were then tested for endotoxin activity using intact cells and extracted LPS (Chembio, UK). The extracted LPS was also visualised for comparison by SDS PAGE and silver staining. Gel images were processed using Image J and Primer 6 statistical software to determine resemblance for each species.

Results & Discussion

Endotoxin detection in seawater.

The total number of Gram negative bacterial cells can be calculated to approximately double in a pollution event (table 1) and since a Gram negative 'bacterium' contains approximately 10² pg of LPS (Ref1 Biocompare, Germany) unpolluted water contains 10.08 pg/100ml, while it is 20.68pg/100ml minimum in polluted bathing waters. Also since 10pg LPS = 0.1EU these levels rise from approx. 0.1 EU/100ml to a minimum of 0.2068 EU/100ml in a pollution event. If the minimum LPS that can be detected using the detection kit is 0.005 EU/ml (Genscript) or 0.5 EU/100ml then the minimum number of bacterial cells that can be considered as a polluted site (0.268 EU/100ml) is below the detection level of the kit. So a positive result, using the current detection system, will only occur with a pollution event of approximately double that of the current bathing water indicator limit. However this needs to be concentrated into 100µl. Our studies show that LPS can successfully be detected at a minimum of 10⁴ bacterial cells/100µl (0.1 OD) for all bacterial isolates, however saltwater gave higher background readings (table2).

Table 1: number of bacteria in clean and polluted event

Bacteria	Unpolluted (cfu/100ml)	Polluted (cfu/100ml)
Total coliforms including <i>E. coli</i>	80	10,000 (1978 Directive)
Enterococci	30	Gram positive
<i>Bacteroides</i>	Zero	600 (minimum, Ref2)
Marine gram negative bacteria	10,000 estimated	>10,000 estimated
Total count	1.008x10 ⁴	2.068x10 ⁴

Table 2: LPS detected using Endotoxin detection kit

Type of bacteria	Optical density	Number of cells/100µl	Diluent
<i>Bacteroides</i>	0.62	6x10 ⁴	water
<i>Bacteroides</i>	1.72	6x10 ⁴	Instant ocean
<i>E. coli</i>	1.17	1x10 ⁵	water
<i>E. coli</i>	1.76	1x10 ⁵	Instant ocean
Gram negative bacteria	1.73	4x10 ⁴	water
Gram negative bacteria	1.80	4x10 ⁴	Instant ocean

***Bacteroides* speciation**

Using indole and sugar utilisation tests for salicin, rhamnose and trehalose, six different *Bacteroides* spp. were isolated from seawater in order of abundance; *B. uniformis*, *B. fragilis/ovatus*, *B. theta/taetamicon/merdae/distasonis*. Two of these have been confirmed as *B. fragilis* spp. using 16S rRNA sequencing. The endotoxin activity of four of these spp. and their extracted LPS is shown in table 3 along with the type culture.

LPS profiling

Extracted *Bacteroides* spp. showed similar banding patterns on SDS PAGE (fig 1).

Table 3: concentration LPS

Bacteria	Cellular LPS (EU/ 10 ⁴ cells)	Extracted LPS (EU/mg)
<i>B. fragilis</i> (isolate 1)	0.126	1.05
<i>B. distasonis</i>	0.126	0.97
<i>B. fragilis</i> (isolate2)	0.124	0.82
<i>B. uniformis</i>	0.139	1.08
NCTC 9343	0.129	1.09

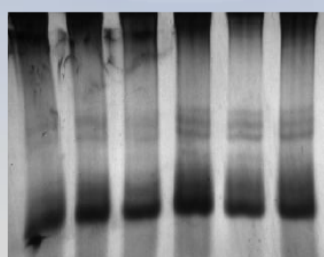


Fig 1: SDS PAGE of extracted LPS from several isolates and type strain (NCTC9343 lane 3).

Conclusions

LPS was successfully tested *in vitro* as a real-time biomarker for sewage pollution indicators but only at increases in indicators which may be just too high for current bathing water standards. Saltwater may interfere with the current detection method. *Bacteroides* specific LPS in seawater was profiled for future development of this as a specific biomarker. Further improvement for a more sensitive and/or specific assay for *Bacteroides* LPS will be needed. Results suggest that the use of LPS has a high potential to be used as a real-time biomarker. The use of *Bacteroides* LPS as a specific biomarker seems promising. Further work is now needed to test simulated polluted water samples and compare with real samples and to develop an improved immunological, and possibly *Bacteroides* LPS specific, detection system.

References

1. BIOCOMPARE: Endotoxins and Their Relevance in R&D Applications - LPS Detection & Removal Methods
- 2: Influence of season, sea state and sampling position on microbial seawater quality monitoring at surfing sites (Unpublished data).

Appendix H

Centre of Research in Translational Biomedicine (CRTB)

research day 5th of April 2011, Plymouth University

Lipopolysaccharide as a real time biomarker of human sewage pathogens in marine bathing waters

Anas Sattar, Graham Bradley, Simon Jackson
 Centre for Research in Translational Biomedicine
 School Of Biomedical and Biological Sciences
 University of Plymouth, United Kingdom



Introduction

Bacterial indicators of human pathogens are important for determining the quality of marine bathing waters. Current methods in use are however retrospective because it is culture based and results take 24-48 hours to be obtained. Since the *Bacteroides* species are strictly present in the intestine of human and warm blooded animals; it represent an excellent prospective bacterial indicator to give an indication of recent sewage pollution in bathing waters. My project focuses on using the Lipopolysaccharide for this purpose. A limulus amoebocyte lysate (LAL) LPS detection kit was calibrated and used to measure the minimum concentration of bacterial LPS of *Bacteroides*, *E. coli* and gram negative bacteria isolates from seawater samples. Lipopolysaccharide (Endotoxin) was successfully tested in vitro as a real-time biomarker. *Bacteroides* specific Lipopolysaccharide in seawater was profiled here to show a possible future development of this biomarker.

Objective

The objective of this research was to develop a positive/negative rapid detection method for *Bacteroides*, *E. coli* and gram negative bacteria LPS and use it as a bacterial indicator for sewage pollution in bathing seawater.

Materials and Methods

Seawater sample collection
 Seawater samples were collected and filtered using a 0.45 µm filter then placed on *Bacteroides* Bile Esculin (BBE) which is a selective medium for the *B. fragilis* group then plates were incubated in an anaerobic incubator for 48 hours then inoculate colonies in to *Bacteroides* phage recovery medium (BPRM) broth to harvest bacterial cells. *E. coli* and Gram negative bacteria were isolated in a similar manner by plating it on a selective media.



Harvesting Bacterial cells
 Bacterial cells were harvested after being inoculated and incubated by centrifuging 2-5 ml of the broth and obtain the pellet which consists of the bacterial cells, which can be used in different assays such as LPS extraction, DNA extraction and LPS detection.



Bacterial speciation

Bacterial speciation performed in order to see if different *Bacteroides* strains give different reactions to the LPS detection kit. Using biochemical tests and sugar utilisation test Salicin, Rhamnose and Trehalose. DNA was (extracted from seven identified *Bacteroides* extracted from seven identified *Bacteroides* and run through the PCR then sent for sequencing to confirm the speciation.



LPS Detection

Using Limulus amoebocyte Lysate, LPS was successfully detected in vitro in a minimum of 10⁴ of bacterial cells of *Bacteroides*, *E. coli* and Gram negative bacteria.



LPS Extraction

LPS was extracted using LPS extraction kit then was run through SDS page electrophoresis to examine the LPS of the seven different isolates. Images obtained from running SDS page were processed using Image J software then Primer 6 software was used to determine resemblance between bands for each strain.



Results

LPS Detection

Table shows data of detection of LPS for *Bacteroides* isolates, *E. coli* and Gram negative with and without seawater. Results show that detection is possible and salt slightly affects the reading for the *E. coli* and gram negative, and significantly for the *Bacteroides*.

Type of bacteria	Dilution	Optical density	Number of colonies	Diluent LAL/ seawater
<i>Bacteroides</i>	10 ⁻⁶	0.623	6x10 ⁶	LAL
<i>Bacteroides</i>	10 ⁻⁶	1.72	6x10 ⁶	Instant ocean
<i>E. coli</i>	10 ⁻⁶	1.166	1x10 ⁵	LAL
<i>E. coli</i>	10 ⁻⁶	1.762	1x10 ⁵	Instant ocean
Gram negative bacteria	10 ⁻⁶	1.733	4x10 ⁶	LAL
Gram negative bacteria	10 ⁻⁶	1.8	4x10 ⁶	Instant ocean

LPS electrophoresis

This figure shows the resemblance the LPS bands of two *Bacteroides* isolates. Indicating that LPS from different *Bacteroides* isolates may be suitable for endotoxin testing.



Conclusion


Results suggest that the use of lipopolysaccharide has a high potential to be used in synergy with current methods in use as a real time biomarker. If the salt can be separated by a mean such as titration.

References

BARON, S. (1995) Medical microbiology 4th ed
 BRADLEY, G., CARTER, J., GAUDIE, D. & KING, A. C. (1996) Distribution of the human faecal bacterium *Bacteroides fragilis* to bacteriophages and their relationship to current sewage pollution indicators in bathing water. *Journal of Applied Microbiology* Symposium 1996, 167, 589-600.
 FRYSGAL, L., NAKI, J. B., LACROIX, S. & STALEY, J. T. (1995) Survival and detection of *Bacteroides* spp., prospective indicator bacteria. *Appl. Environ. Microbiol.* 61, 148-150.
 LAEMMLI, U. K. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* 227, 680-685.

Appendix I

Postgraduate society conference series, Plymouth University, UK, 17th of March 2011 (best poster presentation prize).



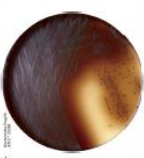
Lipopolysaccharide as a real time Biomarker of human sewage pathogens in marine bathing waters

Anas Sattar, Graham Bradley, Simon Jackson

University of Plymouth, United Kingdom


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Harvesting Bacterial cells
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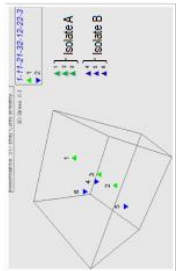
Bacterial speciation
Bacterial speciation performed in order to see if different *Bacteroides* strains give different reactions to the LPS detection kit. Using biochemical tests and sugar utilisation test Salicin, Rhamnose and Trehalose. DNA was (extracted from seven identified *Bacteroides*

Results

LPS Detection
Table shows data of detection of LPS for *Bacteroides* isolates, *E. coli* and Gram negative with and without seawater. Results show that detection is possible and salt slightly affects the reading for the *E. coli* and gram negative, and significantly for the *Bacteroides*.

Type of bacteria	Dilution	Optical density	Number of colonies	Diluent
<i>Bacteroides</i>	10 ⁻¹	0.623	6.10 ⁶	LAL/seawater
<i>Bacteroides</i>	10 ⁻¹	1.72	6.10 ⁶	LAL
<i>E. coli</i>	10 ⁻¹	1.166	1.10 ⁶	Instant ocean
<i>E. coli</i>	10 ⁻¹	1.762	1.10 ⁶	Instant ocean
Gram negative bacteria	10 ⁻¹	1.733	4.10 ⁶	LAL
Gram negative bacteria	10 ⁻¹	1.6	4.10 ⁶	Instant ocean

LPS electrophoresis
This figure shows the resemblance the LPS bands of two *Bacteroides* isolates. Indicating that LPS from different *Bacteroides* isolates may be suitable for endotoxin testing.



Conclusions
Results suggest that the use of lipopolysaccharide has a high potential to be used in synergy with current methods is use as a real time biomarker if the salt can be separated by a mean such as filtration.

References

 1. WHO, 1996. *Water quality guidelines for health*. Geneva: World Health Organization. 228 pp.
 2. WHO, 2003. *Guidelines for drinking-water quality*. Geneva: World Health Organization. 104 pp.
 3. WHO, 2004. *Guidelines for drinking-water quality*. Geneva: World Health Organization. 104 pp.
 4. WHO, 2005. *Guidelines for drinking-water quality*. Geneva: World Health Organization. 104 pp.
 5. WHO, 2006. *Guidelines for drinking-water quality*. Geneva: World Health Organization. 104 pp.
 6. WHO, 2007. *Guidelines for drinking-water quality*. Geneva: World Health Organization. 104 pp.
 7. WHO, 2008. *Guidelines for drinking-water quality*. Geneva: World Health Organization. 104 pp.
 8. WHO, 2009. *Guidelines for drinking-water quality*. Geneva: World Health Organization. 104 pp.
 9. WHO, 2010. *Guidelines for drinking-water quality*. Geneva: World Health Organization. 104 pp.
 10. WHO, 2011. *Guidelines for drinking-water quality*. Geneva: World Health Organization. 104 pp.

Appendix J

***Escherichia coli* strain U 5/41 16S ribosomal RNA, partial sequence**

```
Query 1
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AGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGG 60

Query 61
TAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGA 120

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Sbjct 61
TAACAGGAAGCAGCTTGCTGCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGA 120

Query 121
AACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCA 180

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Sbjct 121
AACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCA 180

Query 181
AGCACAAAGAGGGGGACCTTAGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCT 240

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Sbjct 181
AGCACAAAGAGGGGGACCTTAGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCT 240

Query 241
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|||||
Sbjct 241
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GCAAACTGGAAGTGAAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG 360

|||||
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Query 361
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Query 601
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|||||
Sbjct 661
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Query 721
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|||||
Sbjct 721
GGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATT 780

Appendix K

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The potential of lipopolysaccharide as a real-time biomarker of bacterial contamination in marine bathing water

Anas A. Sattar, Simon K. Jackson and Graham Bradley

ABSTRACT

The use of total lipopolysaccharide (LPS) as a rapid biomarker for bacterial pollution was investigated at a bathing and surfing beach during the UK bathing season. The levels of faecal indicator bacteria *Escherichia coli* (*E. coli*), the Gram-positive enterococci, and organisms commonly associated with faecal material, such as total coliforms and *Bacteroides*, were culturally monitored over four months to include a period of heavy rainfall and concomitant pollution. Endotoxin measurement was performed using a kinetic Limulus Amebocyte Lysate (LAL) assay and found to correlate well with all indicators. Levels of LPS in excess of 50 Endotoxin Units (EU) mL⁻¹ were found to correlate with water that was unsuitable for bathing under the current European regulations. Increases in total LPS, mainly from Gram-negative indicator bacteria, are thus a potential real-time, qualitative method for testing bacterial quality of bathing waters.

Key words | faecal indicator bacteria, LPS, rapid method, sewage pollution, surfing/bathing water

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INTRODUCTION

The quality of beaches in the UK and Europe is governed by the European Bathing Water Directive (EU 2006) which sets legislation and defines standards that all beaches must comply with in order to be considered as a designated bathing beach. Faecal Indicator Bacteria (FIB), namely *Escherichia coli* and enterococci, are important indicators for determining the quality of marine bathing waters as they can eliminate the need for expensive and time consuming testing for pathogenic bacteria and viruses (Lucena *et al.* 1994). The FIB previously used were total coliforms, faecal coliforms, *E. coli* and faecal streptococci as these microorganisms can provide a general indication of faecal pollution; these were later revised and coliforms were substituted by enterococci. However, current quantitative culture-based FIB methods have drawbacks (Borrego *et al.* 1983; Anderson *et al.* 2005; Field & Samadpour 2007) as these indicators have a tendency to multiply in bathing waters and the results are retrospective, taking at least 24–48 h to inform regulatory bodies. Marine recreational water users themselves really need an instant, qualitative indication of possible bacterial pollution events

in order to make an informed decision on whether to undertake an activity. The term lipopolysaccharide (LPS) is often used interchangeably with endotoxin and is the major component of the outer membrane of all Gram-negative bacteria (Raetz 1990; Rietschel *et al.* 1994). LPS has been targeted by previous studies for estimating total biomass and testing potable water (Jorgensen *et al.* 1976, 1979; Watson *et al.* 1977; Evans *et al.* 1978; Haas *et al.* 1983) but not as a potential biomarker for bacteria-polluted bathing water, possibly due to background levels from Gram-negative marine species.

Studies conducted by Fiksdal *et al.* (1985), Allsop & Stickler (1985) and Kreader (1995) have suggested the use of *Bacteroides* species as an excellent bacterial indicator candidate since it is shed in high numbers in faeces and is unlikely to reproduce in seawaters. However, because of its anaerobic nature and consequent difficult culture requirement, it has always been overlooked by legislators. *Bacteroides* species inhabit the intestines of human and warm-blooded animals where they comprise 30–50% of normal faecal matter in humans (Isar

et al. 2006) and they are present in bathing waters when polluted with poorly treated sewage from urban discharge or runoff from farms, hence *Bacteroides* was included in this study.

Therefore, the aim of this study was to measure the *total* LPS levels in seawater and correlate them with the current culture-based methods for determining this water quality using bacterial indicators such as *E. coli* and *Bacteroides*. This was undertaken at a surfing/bathing beach during the UK bathing season, including a high rainfall pollution event.

MATERIALS AND METHODS

Seawater sampling regime

Challaborough beach was chosen as a popular bathing, body boarding and surfing beach located in the South Hams district of Devon, UK (Latitude: 50.287159, Longitude: -3.899052). It is a horseshoe-shaped bay divided by a small stream that runs from a valley down into the sea (Figure 1).

Challaborough village comprises two small fixed caravan sites and a few private houses which end just beside

the beach. This beach was chosen because it is divided into two distinct areas: bathing and surfing.

Composite samples, comprising the four stations from each area, were collected on 10 occasions over the summer of 2012 at Challaborough beach starting from early June to the beginning of September (UK bathing season), in addition to water samples from the stream. Samples were usually taken once a week with a random timing for low and high tide, except for one sample in which heavy rainfall and high level of runoff had been observed and which was considered as the 'polluted' sample (15/08/2012 sample in Figure 3). Samples were collected in 500 mL disposable, sterile, screw-capped wide-mouth pots for the bacteriological investigation whilst 50 mL endotoxin-free Falcon™ tubes (BD Biosciences, UK) were used to collect water samples for LPS detection. Shallow water samples were collected from the surface of the water whilst deeper water samples were taken from approximately 1 m depth. Sediment samples were also collected in similar pots from the shallow water area for both bathing and surfing areas. Samples were immediately taken to the laboratory and processed within 3 h. Samples for LPS detection were aliquoted into endotoxin-free glass tubes (Lonza, UK) and preserved at -20 °C until assay.

Seawater sample filtration

Water samples were filtered aseptically through a 0.45 µm membrane (Whatman, UK) and placed on appropriate media; *Bacteroides* Bile Esculin (BBE) (Livingston *et al.* 1978), Membrane Lauryl Sulphate Broth, and Slanetz and Bartley agar (Oxoid, UK) to isolate *Bacteroides*, *total* coliforms/*E. coli* and enterococci, respectively. Appropriate volumes of the samples were aseptically filtered in duplicate using a vacuum pump attached to the water filtration system. A 1:10 dilution of the sediment samples (2 g) was prepared in sterile simulated sea water (Instant Ocean, Underworld, UK) (18 mL) and placed in stomacher bags that were placed in a stomacher (Seward Lab, UK) for 2 minutes, and left to settle for 10 minutes. Then, 10 mL of the supernatant was filtered in a manner similar to the method described for water sample filtration above. BBE agar cultures were incubated in an anaerobic incubator (Don Whitley, UK) at 37 °C for 48–72 h, Slanetz and Bartley



Figure 1 | Topography of Challaborough beach at low tide and the surrounding caravan site. A, B, C and D represent sampling stations for shallow water and sediment at the bathing area. E, F, G and H represent sampling stations of approximately 1 m depth water sampling in the bathing area. I, J, K and L represent sampling stations for shallow water and sediment at the surfing area. M, N, O and P represent sampling stations of approximately 1 m depth water sampling in the surfing area. Q represents the sampling site for stream water.

agar was incubated aerobically at 35 °C for 4 h then at 44 °C for 44 h, and Membrane Lauryl Sulphate total coliforms cultures were incubated aerobically at 35 °C for 24–48 h and for *E. coli* incubated aerobically at 35 °C for 4 h, then at 44 °C for 44 h.

Enumeration and isolation of bacterial colonies

The numbers of colony-forming units (CFU) were calculated and numbers were expressed as CFU 100 mL⁻¹ of water or per g⁻¹ of sediment.

Determination of total endotoxin concentration

In order to determine whether there was a correlation between the total LPS concentration in bathing water and the number of CFU of the bacterial indicators, a kinetic-QCL™ LAL assay (Lonza, UK) was conducted to determine the LPS concentration in the water samples. Briefly, the principle of the kinetic-QCL™ LAL assay is that the endotoxin sample from Gram-negative bacteria is mixed with Limulus Amebocyte Lysate (LAL, extracted from horseshoe crab *Limulus polyphemus*) substrate in appropriate condition and monitored over a period of time for the appearance of a colour change due to endotoxin presence. Reaction time is the time that a colour change (yellow) reaches an optical density absorbance of 0.2; this reaction time is inversely proportional to the concentration of endotoxin and the concentration of unknown samples is determined from standard curve.

A composite sample of the 10 water samples from the stream in addition to each area, i.e. shallow or deep (S or D), bathing or surfing (bathe or surf), was prepared. Four dilutions (undiluted sample, 1/10, 1/100 and 1/1000) from each composite sample were run for their total LPS activity using the kinetic-QCL™ method to investigate whether different sampling areas possess different LPS levels. In addition, 10 individual water samples from the bathing area were assayed for their endotoxin activity to assess whether the total LPS levels correlate to the number of *E. coli* and *Bacteroides* and followed a similar trend to these bacterial numbers. Water samples were diluted with endotoxin-free water and assayed for their endotoxin activity using a kinetic LAL assay kit (Kinetic-QCL™; Lonza, UK)

according to the manufacturer's instructions. Endotoxin from *E. coli* O55:B5 (provided with the kit) was used as a standard to calibrate the assay as described by the manufacturer's instructions. Appropriate serial dilutions were prepared from the endotoxin standard and samples from 50 to 0.005 Endotoxin Units (EU)/mL. The US Food and Drug Administration (FDA) defined an EU as the endotoxin activity of 0.2 ng of Reference Endotoxin Standard depending on the source of endotoxin (Liebers et al. 2006). Presently, the conversion rate of the current reference standard used by the FDA (EC-6) is 10 EU/ng. Samples and standard endotoxins in duplicate were loaded into endotoxin-free 96-well plates (Costar, Corning, USA) and incubated for 10 minutes at 37 °C in a pre-warmed plate reader (BioWhittaker elx808, BioTek, UK). The default template parameters of WinKQCL Endotoxin detection and analysis software version 3.0.1 (Lonza) were set to 40 readings absorbance with a 405 nm measurement filter. Concentrations of unknown samples were calculated using the values of the standard curve to give a quantitative endotoxin EU value. All tips (Fisher Scientific, UK) and glass dilution tubes (Lonza, USA) used in each experiment were certified as endotoxin-free. As a quality assessment of the assay, a spike and recovery experiment using a known quantity of standard endotoxin was performed to investigate any inhibition/enhancement when assaying for endotoxin in seawater.

Statistical analysis

Statistical analysis was performed using Microsoft Office Excel 2010 and Minitab® version 16.1.1 (Kruskal-Wallis test and one-way analysis of variance). LAL assay concentrations were calculated using WinKQCL Endotoxin Detection and Analysis Software Version 3.0.1 (Lonza).

RESULTS

Bacterial enumeration and total LPS estimation

The mean CFU in the 10 composite samples of the four areas for *Bacteroides*, *E. coli*, enterococci and total coliforms ($n = 10$) over the sampling period are shown in Figure 2. The number of indicator bacteria generally

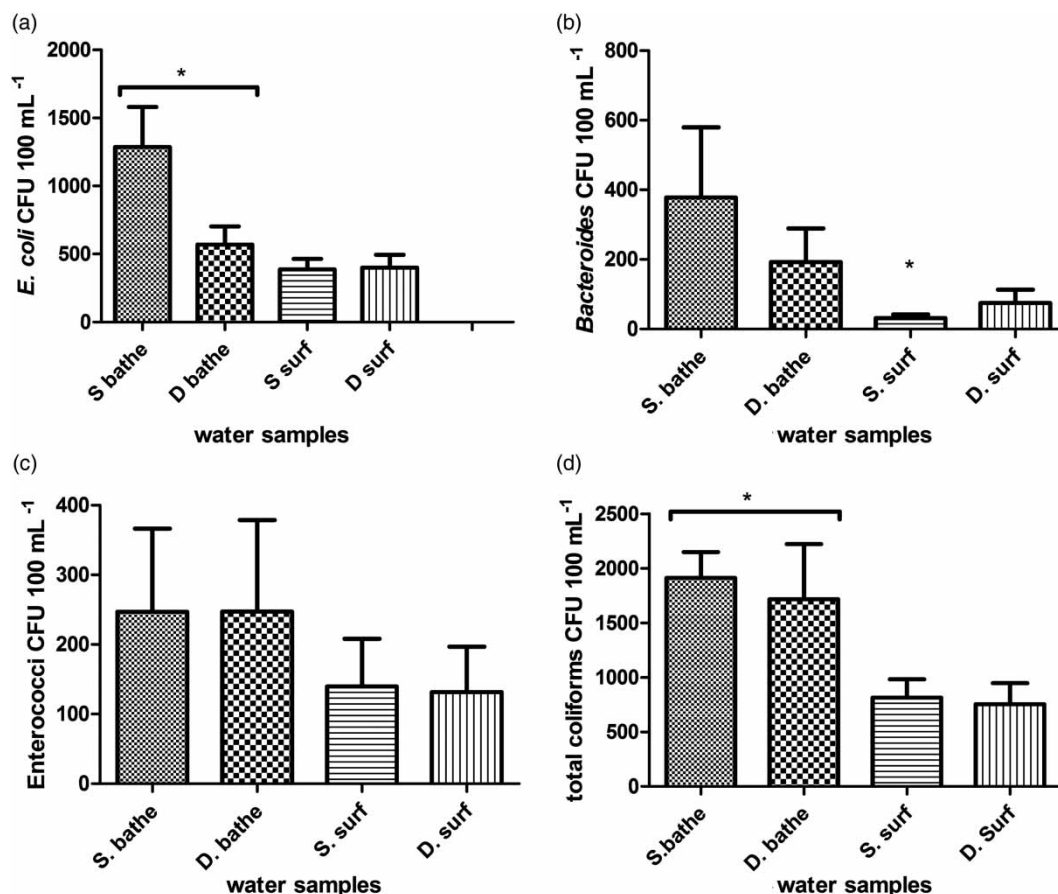


Figure 2 | Mean number of CFU 100 mL⁻¹ bacteria in 10 composite bathing water samples for four areas. (a) *E. coli*, (b) *Bacteroides* species, (c) enterococci and (d) total coliforms. S. bathe represents shallow water samples collected from the bathing area, D. Bathe represents deep water samples collected from the bathing area, S. surf represents shallow water samples collected from the surfing area, and D. surf represents deep water samples collected from the surfing area. $n = 10$. Bars show standard error of means. * means *E. coli* $p < 0.05$ versus S. surf and D. surf; *Bacteroides* $p < 0.05$ versus S. bathe; Total coliforms $p < 0.05$ versus S. surf and D. surf.

showed that the bathing area was more contaminated than the surfing area. There was a significant difference in the number of CFU of *E. coli* ($p = 0.03$) and total coliforms ($p = 0.01$) when comparing the surfing and the bathing areas.

Bacteroides results only showed a significant difference between S. surf and S. bathe areas. Enumerated enterococci showed no significant difference between the bathing and the surfing areas, however, the numbers of CFUs were still higher than in the bathing area. A 95th and 90th percentile was calculated according to the recommended formula by the 2006 European directive; results showed that all the water samples from both bathing and surfing areas were classified as 'poor' (data not shown). Sediment samples showed extremely low numbers of bacteria; the sediment

consists of small rocks and sand grains which tend to have a poor surface for bacterial attachment and also therefore as a bacterial reservoir (Harrison 2012).

The mean LPS concentration over the sampling period for each area is shown in Figure 3 where LPS concentration varied between 34 and 135 EU mL⁻¹. There were highly significant differences among all the sampling sites.

When examined over the time period, a distinct increase in contamination was shown for all indicators after a period of heavy rainfall and concomitant runoff (data shown for *Bacteroides* and *E. coli* only, Figure 4). Endotoxin levels followed a similar trend to the number of FIB CFUs showing the highest increase at the polluted run-off event (15/08/12) and returning to threshold levels within the next sampling period (7 days).

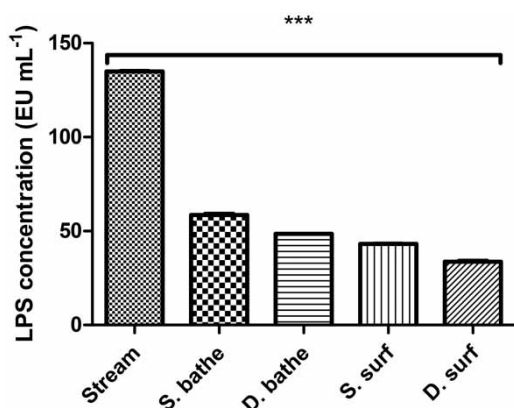


Figure 3 | Mean LPS concentrations of 10 composite water samples from five areas. A one-way analysis of variance was conducted to evaluate the difference in LPS concentrations among different water sampling areas using Tukey's Multiple Comparison Test. *** means $p < 0.001$ among all sampling areas. Stream represents water samples collected directly from the stream area, S. bathe represents shallow water samples collected from the bathing area, D. bathe represents deep water samples collected from the bathing area, S. surf represents shallow water samples collected from the surfing area, D. surf represents deep water samples collected from the surfing area. $n = 4$. Error bars show standard error of means.

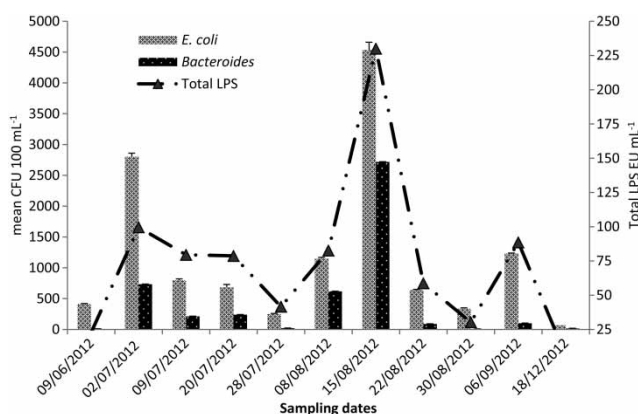


Figure 4 | Mean number of *Bacteroides* and *E. coli* CFU 100 mL⁻¹ and total endotoxin concentration EU mL⁻¹ in water samples ($n = 4$) of the shallow bathing area during the sampling periods. The dotted line represents the cut-off of LPS concentration above which readings are considered as unsuitable water for bathing.

Good correlations between the number of indicator bacteria and concentrations of total LPS were obtained (Figure 5). The European directive threshold value for *E. coli* is also shown in Figure 5(a) and is converted to EU of LPS.

The use of undiluted seawater samples indicated that there was inhibition of the LAL assay. To confirm the inhibition, a spike-and-recovery assessment was performed by spiking artificial seawater (Instant Ocean) to a final

concentration of 0.5 EU mL⁻¹ with appropriate controls and performing the LAL assay. The recovery of LPS was over 10-fold lower at undiluted levels but this inhibition was removed at dilutions of more than one in 10. Consequently, all seawater samples were diluted 1/100 for LPS measurements.

DISCUSSION AND CONCLUSIONS

Testing the quality of marine bathing waters is an essential procedure to ensure compliance with EU legislation and the health of bathers and watersports performers. However, a real-time cost effective method is needed as current culture-based methods are time consuming with retrospective results reflecting the water quality status at least 24–48 h previous. Results from this study have shown that total LPS can be used as a rapid, qualitative biomarker taking approximately 1.5 h and can be optimised to give qualitative results in 30 minutes to detect levels above a calculated threshold of 50 EU mL⁻¹ of LPS in marine bacterial and faecal contamination. Studies conducted previously have explored the use of LPS as an indicator for testing contamination in drinking and other water. Jorgensen *et al.* (1976) ran a pilot study to explore the possibility of using a LAL assay to estimate the concentration of endotoxins in potable and reclaimed, advanced waste treatment waters and showed that chlorination of water interferes with the assay. Watson *et al.* (1977) used three techniques including the determination of LPS concentration to estimate the biomass and number of bacteria in marine water. That study showed that LPS can be related to the number of bacteria and suggested a factor to convert LPS to bacterial carbon. LPS concentration in water supplies from a stream water correlating with the culture-based bacterial count of coliforms, enteric, Gram-negative and heterotrophic bacteria was investigated by Evans *et al.* (1978) using the gel clot and a spectrophotometric LAL assay. This showed that the gel clot method was less sensitive and less reproducible than the spectrophotometric assay; Evans *et al.* (1978) study also suggested the continued refining of the LAL assay for implementation in water quality investigation.

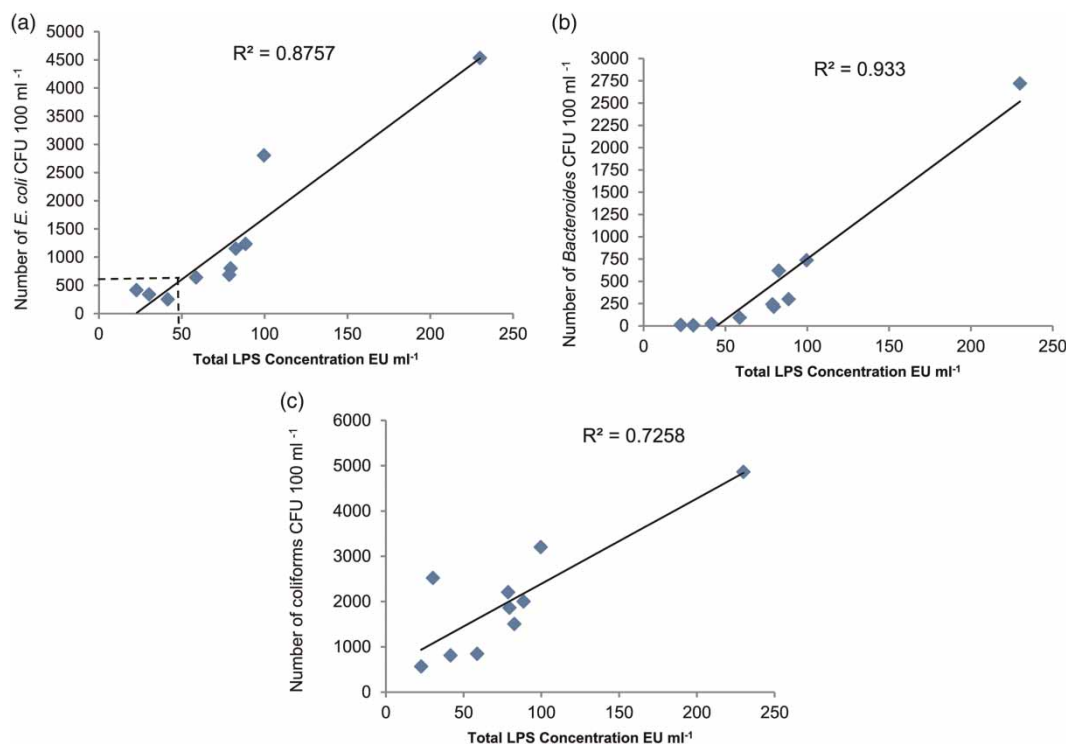


Figure 5 | Pearson correlation of 10 composite water sample means for CFU and total LPS at the shallow bathing area (S. bathe) (a) *E. coli*, Pearson correlation of LPS and *E. coli* = 0.936, $p < 0.001$ (b) *Bacteroides*, Pearson correlation of LPS and *Bacteroides* = 0.954, $p < 0.001$ and (c) total coliforms, Pearson correlation of LPS and total coliforms = 0.852, $p < 0.05$. A threshold as set by the European directive and the corresponding EU/mL of endotoxin is highlighted with the dotted line where appropriate.

In the current study, a more accurate and highly sensitive kit was used to investigate the bacteriological LPS quality of recreational bathing seawater. The kinetic-QCL™ is a highly sensitive method that can detect LPS down to 0.005 EU mL⁻¹. Results showed that total LPS concentration correlates very well with the current bacterial indicator, *E. coli* and with *Bacteroides*, and slightly less with total coliforms (Figure 5), all including a pollution event after heavy rainfall. Increases in LPS also appeared to dissipate from the bathing water within the time period of the next sample (7 days) and probably within the 70 h by the methods described by Shibata *et al.* (2009). LPS molecules are shed from the Gram-negative bacterial cell wall when the environmental factors are not suitable for these cells or when infected with bacteriophages (Fuhrman 2000; Nagata 2000). LPS is being constantly removed from seawater by flagellates (Shibata *et al.* 2009). The LPS threshold at which seawater can be considered as polluted or unsuitable for bathing was determined based on the current legislated number for a 'sufficient' level of bacterial

indicators in the European directive 2006 guide for coastal bathing water quality. Using Figure 5, the authors estimate the corresponding LPS concentration at these limits to be equivalent to 50.3 EU mL⁻¹. This level could be set for a future real-time qualitative bathing water test kit development.

Marine bathing water represents a sanctuary for eukaryotes and prokaryotes, including indigenous Gram-negative bacteria. When estimating the total LPS from marine bathing waters, part of the total LPS measurement is due to the presence of 'background indigenous Gram-negative bacteria' which will affect the estimation of LPS using the LAL assay. However, many studies have shown that the major bacterial species present in seawater is characterised by low endotoxin virulence because low acylation and phosphorylation are encountered in marine Gram-negative bacteria (Ramos *et al.* 2001; Kraskova *et al.* 2004; Leone *et al.* 2007). The presence of low acylation – penta- and tetra-acyl species – and phosphorylation in the lipid A structure signifies low immunological

activity compared to endotoxically active molecules (Rietschel *et al.* 1994) and is also an indication of low activity with the LAL assay. Hence, even when there is an increase in the background levels it is likely to induce a low reactivity in the LAL assay. Collected water samples processed in this study have never shown a false positive in the LAL assay, and low CFU numbers of FIB (especially *E. coli*) were always accompanied by low LAL activity. The background level of LPS was measured in an excellent quality bathing water sample and results showed it was 6 EU mL⁻¹. However, there is a possibility of occasionally observing false positives, events above 50 EU mL⁻¹, in the absence of FIB. This may perhaps be true due to, for example, eutrophication (Nixon 1995) in seawater that allows marine Gram-negative bacteria to thrive. A less plausible source is from cyanobacterial blooms and an increase in vibrio densities. Although these increases in total LPS would be related to a non-faecal pollution event, this can still be useful as an indicator for the presence of these blooms which have been shown to cause serious human health problems such as gastrointestinal tract infections, cyanobacterial intoxications and even cholera (Morris & Acheson 2003; Dietrich *et al.* 2008). Our immunological studies (unpublished data) have shown that an increase in LPS levels in contaminated bathing water is directly linked to an increase in human cell line pro-inflammatory cytokines and the production of these cytokines was dose-dependent on LPS levels. Hence the LAL test has an advantage in detecting total LPS levels even in a non-faecal contamination event.

The quantitative kinetic-QCL™ assay was performed in approximately 1.5 h when using a 10-fold range of LPS standards 0.005–50 EU mL⁻¹ and approximately 30 minutes to detect 0.5 EU and above, which is equivalent to 50 EU mL⁻¹ after 100-fold dilution. This method could potentially be optimised as an endpoint chromogenic assay, manufactured and available for untrained bathers as a real-time, qualitative, single-use kit to examine the bacterial quality of bathing waters. The LAL method is not an attempt to replace the applicability of current culture or future PCR methods as these are a valuable method used in microbial source tracking but could be used as part of a 'tool box' approach to water quality management.

Whilst background levels of endogenous LPS appeared to remain low and stable during this study, a *specific* LPS assay for one indicator would be preferable. The authors are working on developing a more specific biomarker involving LPS of *Bacteroides* as real time biomarker using ELISA assay.

In conclusion, a rapid method has been developed to screen the bacterial quality of marine bathing water and has the potential to be developed and used as a qualitative, on-the-spot test by bathers and beach goers, based on an excellent correlation between the culture-based method and total LPS determination.

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REFERENCES

- Allsop, K. & Stickler, D. J. 1985 An assessment of *Bacteroides fragilis* group organisms as indicators of human faecal pollution. *J. Appl. Microbiol.* **58**, 95–99.
- Anderson, K. L., Whitlock, J. E. & Harwood, V. J. 2005 Persistence and differential survival of fecal indicator bacteria in subtropical waters and sediments. *Appl. Environ. Microbiol.* **71**, 3041–3048.
- Borrego, J. J., Arraba, F., De Vicente, A., Gomez, L. F. & Romero, P. 1985 Study of microbial inactivation in the marine environment. *J. Water Pollut. Control Fed.* **55**, 297–302.
- Dietrich, D., Fischer, A., Michel, C. & Hoeger, S. J. 2008 Toxin mixture in cyanobacterial blooms – a critical comparison of reality with current procedures employed in human health risk assessment. In: *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs* (H. K. Hudnell, ed.). Springer, New York, pp. 885–912.
- EU 2006 Directive 2006/7/EC of the European parliament and of the council of 15 February 2006 concerning the management of bathing water quality and repealing directive 76/160/EEC. *Official Journal of the European Union* **L64**, 37–52.
- Evans, T. M., Schillinger, J. E. & Stuart, D. G. 1978 Rapid determination of bacteriological water quality by using limulus lysate. *Appl. Environ. Microbiol.* **35**, 376–382.
- Field, K. G. & Samadpour, M. 2007 Fecal source tracking, the indicator paradigm, and managing water quality. *Water Res.* **41**, 3517–3538.

- Fiksdal, L., Maki, J. S., Lacroix, S. J. & Staley, J. T. 1985 Survival and detection of *Bacteroides* spp., prospective indicator bacteria. *Appl. Environ. Microbiol.* **49**, 148–150.
- Fuhrman, J. A. 2000 Impact of viruses on bacterial processes. In: *Microbial ecology of the oceans*. (D. L. E. Kirchman, ed.). Wiley-Liss, New York, pp. 327–350.
- Haas, C. N., Meyer, M. A., Paller, M. S. & Zapkin, M. A. 1983 The utility of endotoxins as a surrogate indicator in potable water microbiology. *Water Res.* **17**, 803–807.
- Harrison, D. 2012 *A Student's Guide to the Seashore*. Emerald Group Publishing Limited, Bingley, UK.
- Isar, J., Agarwal, L., Saran, S. & Saxena, R. K. 2006 Succinic acid production from *Bacteroides fragilis*: Process optimization and scale up in a bioreactor. *Anaerobe* **12**, 231–237.
- Jorgensen, J. H., Lee, J. C. & Pahren, H. R. 1976 Rapid detection of bacterial endotoxins in drinking water and renovated wastewater. *Appl. Environ. Microbiol.* **32**, 347–351.
- Jorgensen, J. H., Lee, J. C., Alexander, G. A. & Wolf, H. W. 1979 Comparison of Limulus assay, standard plate count, and total coliform count for microbiological assessment of renovated wastewater. *Appl. Environ. Microbiol.* **37**, 928–931.
- Krasikova, I. N., Kapustina, N. V., Isakov, V. V., Dmitrenok, A. S., Dmitrenok, P. S., Gorshkova, N. M. & Solov'eva, T. F. 2004 Detailed structure of lipid A isolated from lipopolysaccharide from the marine proteobacterium *Marinomonas vaga* ATCC 27119T. *Eur. J. Biochem.* **271**, 2895–2904.
- Kreader, C. A. 1995 Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. *Appl. Environ. Microbiol.* **61**, 1171–1179.
- Leone, S., Silipo, A., Nazarenko, E. L., Lanzetta, R., Parrilli, M. & Molinaro, A. 2007 Molecular structure of endotoxins from Gram-negative marine bacteria: an update. *Mar. Drugs* **5** (3), 85–112.
- Liebers, V., Brüning, T. & Raulf-Heimsoth, M. 2006 Occupational endotoxin-exposure and possible health effects on humans (review). *Am. J. Ind. Med.* **49**, 474–491.
- Livingston, S. J., Kominos, S. D. & Yee, R. B. 1978 New medium for selection and presumptive identification of the *Bacteroides fragilis* group. *J. Clin. Microbiol.* **7**, 448–453.
- Lucena, F., Lasobras, J., McIntosh, D., Forcadell, M. & Jofre, J. 1994 Effect of distance from the polluting focus on relative concentrations of *Bacteroides fragilis* phages and coliphages in mussels. *Appl. Environ. Microbiol.* **60**, 2272–2277.
- Morris, J. G. & Acheson, D. 2003 Cholera and other types of Vibriosis: a story of human pandemics and oysters on the half shell. *Clin. Infect. Dis.* **37**, 272–280.
- Nagata, T. 2000 Production mechanisms of dissolved organic matter. In: *Microbial ecology of the oceans*. (D. L. E. Kirchman, ed.). John Wiley & Sons, New York, pp. 121–152.
- Nixon, W. 1995 Coastal marine eutrophication—A definition, social causes, and future concerns. *Ophelia* **41**, 199–219.
- Raetz, C. R. H. 1990 Biochemistry of endotoxins. *Annu. Rev. Biochem.* **59**, 129–170.
- Ramos, J. L., Gallegos, M. A.-T., Marqués, S., Ramos-González, M.-I., Espinosa-Urgel, M. & Segura, A. 2001 Responses of Gram-negative bacteria to certain environmental stressors. *Curr. Opin. Microbiol.* **4**, 166–171.
- Rietschel, E. T., Kirikae, T., Schade, F. U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A. J., Zähringer, U., Seydel, U. & Di Padova, F. 1994 Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J.* **8**, 217–225.
- Shibata, A., Yasui, H. & Fukuda, H. 2009 Fate of the bacterial cell envelope component, lipopolysaccharide, that is sequentially mediated by viruses and flagellates. *Coast. Mar. Sci.* **33**, 39–45.
- Watson, S. W., Novitsky, T. J., Quinby, H. L. & Valois, F. W. 1977 Determination of bacterial number and biomass in the marine environment. *Appl. Environ. Microbiol.* **33**, 940–946.